



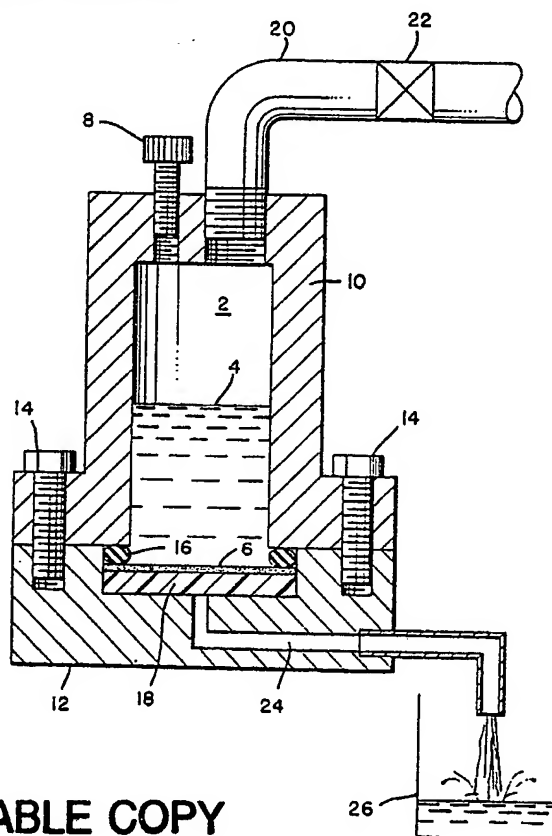
INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ³ : B01D 13/00	A1	(11) International Publication Number: WO 86/ 00238 (43) International Publication Date: 16 January 1986 (16.01.86)
(21) International Application Number: PCT/US85/01161 (22) International Filing Date: 19 June 1985 (19.06.85) (31) Priority Application Numbers: 622,502 622,690 (32) Priority Dates: 20 June 1984 (20.06.84) 20 June 1984 (20.06.84) (33) Priority Country: US (71) Applicant: THE LIPOSOME COMPANY, INC. [US/ US]; One Research Way, Princeton Forrestal Center, Princeton, NJ 08540 (US). (72) Inventors: CULLIS, Pieter, R. ; 1329 Walnut Street, Vancouver, British Columbia V6J 3R2 (CA). HOPE, Michael, J. ; 3550 West 11th Avenue, Vancouver, Brit- ish Columbia V6R 2K2 (CA). BALLY, Marcel, B. ; 5516 Corvette Crescent, Vancouver, British Columbia V6T 1M8 (CA).	(74) Agent: KLEE, Maurice, M.; 325 Myrtle Avenue, Bridgeport, CT 06604 (US). (81) Designated States: AT (European patent), BE (Euro- pean patent), CH (European patent), DE (European patent), DK, FR (European patent), GB (European patent), IT (European patent), JP, LU (European pa- tent), NL (European patent), SE (European patent). Published With international search report.	

(54) Title: EXTRUSION TECHNIQUES FOR PRODUCING LIPOSOMES

(57) Abstract

Extrusion techniques for producing populations of liposomes having a substantially unimodal size distribution and for producing unilamellar liposomes. The unimodal size distribution is achieved by repeatedly passing previously formed liposomes through one or a plurality of filters, all of which have the same pore size. The unilamellar liposomes are produced by using a filter which has a pore size equal to or less than about 100 nm. In accordance with other aspects of the disclosure, liposomes are prepared directly from a lipid powder or pellet and buffer without the use of any solvents, detergents or other extraneous materials.



BEST AVAILABLE COPY

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	GA	Gabon	MR	Mauritania
AU	Australia	GB	United Kingdom	MW	Malawi
BB	Barbados	HU	Hungary	NL	Netherlands
BE	Belgium	IT	Italy	NO	Norway
BG	Bulgaria	JP	Japan	RO	Romania
BR	Brazil	KP	Democratic People's Republic of Korea	SD	Sudan
CF	Central African Republic	KR	Republic of Korea	SE	Sweden
CG	Congo	LI	Liechtenstein	SN	Senegal
CH	Switzerland	LK	Sri Lanka	SU	Soviet Union
CM	Cameroon	LU	Luxembourg	TD	Chad
DE	Germany, Federal Republic of	MC	Monaco	TG	Togo
DK	Denmark	MG	Madagascar	US	United States of America
FI	Finland	ML	Mali		
FR	France				

-1-

5

EXTRUSION TECHNIQUES FOR PRODUCING LIPOSOMESBACKGROUND OF THE INVENTION1. Field of the Invention

10 This invention relates to liposomes and in particular to extrusion techniques for the rapid production of unilamellar liposomes and for the production of liposomes having defined size distributions.

2. Description of the Prior Art

15 As is well known in the art, liposomes are closed vesicles having a lipid bilayer membrane surrounding an aqueous core. In general, liposomes of the following three types have been produced: 1) multilamellar vesicles (MLVs) wherein each vesicle includes multiple concentric bilayer membranes stacked one inside
20 the other in an onion skin arrangement; 2) small unilamellar vesicles (SUVs) having only one bilayer membrane per vesicle and having diameters ranging up to about 50 nm; and 3) large unilamellar vesicles (LUVs), again having only one bilayer membrane per vesicle, but in this case having diameters greater than
25 about 50 nm and typically on the order of 100 nm and above.

A review of these three types of liposomes, including methods for their preparation and various uses for the finished liposomes, can be found in the text Liposomes, Marc J. Ostro, ed., Marcel Dekker, Inc., New York, 1983, the pertinent portions of which are
30 incorporated herein by reference. See also Szoka, Jr., et al., Ann. Rev. Biophys. Bioeng., 9:467 (1980), the pertinent portions of which are also incorporated herein by reference.

Other types of liposomes which have been developed include stable plurilamellar vesicles (SPLVs), monophasic vesicles (MPVs),
35 and steroidal liposomes. Descriptions of these vesicles and

-2-

5 methods for preparing them can be found in copending and commonly assigned U.S. Patent Applications Serial Nos. 476,496, 521,176, and 599,691, filed on March 24, 1983, August 8, 1983, and April 12, 1984, respectively, the pertinent portions of which are incorporated herein by reference.

10 One of the primary uses for liposomes is as carriers for a variety of materials, such as, drugs, cosmetics, diagnostic reagents, bioactive compounds, and the like. Liposomes are also widely used as scientific models for naturally occurring biological membrane systems.

15 In connection with each of these uses, it is important to have available populations of liposomes which have defined mean diameters and defined size distributions about those means. More particularly, it is important to have available populations of liposomes which have a substantially unimodal distribution about a selected mean diameter.

20 In terms of commercial applications, and in particular, pharmaceutical applications, such populations are needed to enhance the effectiveness and safety of liposome encapsulated drugs and similar materials. Moreover, the availability of accurately defined populations of liposomes would make it significantly easier to obtain approval for liposome-containing preparations from such regulatory agencies as the United States Food and Drug Administration. In terms of other liposome applications, including
25 scientific investigations, the ready availability of well-characterized populations of liposomes would lead to more standardized products and repeatable experiments.

The present invention relates to improved methods for the production of liposomes. In particular, the invention relates to:
30 1) an improved method for producing unilamellar liposomes of both the large and small types; and 2) an improved method for producing liposomes having defined size distributions.

Prior to the present invention, large unilamellar liposomes (LUVs) were commonly produced by one of the following three
35 methods: 1) reverse-phase evaporation, 2) detergent dilution, and

-3-

3) infusion procedures using various solvents. See Liposomes, supra, Ch. 1, pages 37-44.

5 In the reverse-phase evaporation technique, an aqueous buffer is introduced into a mixture of phospholipid and an organic solvent to produce "inverted micelles," i.e., droplets of water stabilized in the organic solvent by being surrounded by a phospholipid monolayer. Evaporation of the solvent causes the micelles to coalesce and form the desired liposomes. See, for example, Szoka, Jr., et al., Proc. Natl. Acad. Sci. USA, 75:4194
10 (1978); and U.S. Patent 4,235,871 to Papahadjopoulos et al.

In the detergent dilution approach, lipid, detergent and an aqueous solution are mixed together and sonicated to form the desired vesicles. Separation techniques, such as, gel filtration, are then used to remove the detergent and thus produce the finished liposomes.
15

In the infusion procedures, lipid is dissolved in a solvent, e.g., pentane or diethyl ether, and the lipid-solvent solution is infused into an aqueous solution under conditions that cause the solvent to vaporize and thus produce the desired liposomes. See, for example, Deamer, Annals New York Academy of Sciences, 308: 250-258 (1978).
20

Other techniques which have been used to produce LUVs include fusion techniques whereby a population of SUVs is treated so as to cause individual SUVs to fuse with each other to form LUVs. For example, U.S. Patent 4,078,052 to P. Demetrios Papahadjopoulos describes a technique wherein calcium ions are used to fuse SUVs into cochleate cylinders, and the cylinders are then treated with a calcium chelating agent such as EDTA to form the desired LUVs. Rapid freezing of SUVs, followed by slow thawing, has also been used to produce LUVs by fusion. See, for example, U.Pick, Archives of Biochemistry and Biophysics, 212:186 (1981).
25
30

With regard to the production of SUVs, as with LUVs, a variety of techniques have been employed in the past. See Liposomes, supra, Ch. 1, pages 33, 36. The earliest technique involved sonication to clarity of a suspension of lipid in an aqueous solution using a probe or bath sonication unit. Other techniques
35

-4-

have included infusion procedures along the lines of those used for producing LUVs but with ethanol as the solvent (see S. Batzri and E. Korn, Biochimica et Biophysica Acta, 298:1015 (1973)), and a technique employing multiple passes of MLVs through a French press operated at a pressure of 20,000 psi (see, for example, Hamilton, Jr., et al., Journal of Lipid Research, 21:981 (1980); and Barenholz, et. al., FEBS Lett., 99:210 (1979)).

In addition to the basic techniques used to produce liposomes, various ancillary techniques have been developed for post-preparation treatment of liposomes to improve their properties. In particular, as discussed more fully below, many of the LUV techniques described above have required sizing of the finished liposomes by filtration using, for example, a series of polycarbonate filters. See Liposomes, *supra*, Ch. 1, pages 37-39, 45; and Szoka, et al., Biochimica et Biophysica Acta, 601:559 (1980). Series of polycarbonate filters have also been used to size MLVs. See F. Olson, et al., Biochimica et Biophysica Acta, 557:9 (1979), and Bosworth, et al., Journal of Pharmaceutical Sciences, 71:806 (1982).

Although each of the foregoing techniques can be used to produce liposomes, none of these techniques are totally satisfactory. For example, each of the commonly used LUV techniques involves combining the components making up the liposome with a lipid solubilizing agent, i.e., either an organic solvent or a detergent. As is well known in the art, solvents and detergents can adversely effect many materials, such as enzymes, which one may want to encapsulate in liposomes, and thus these techniques cannot be used with these materials. Also, in applications such as the generation of drug carrier systems, the possible presence of these potentially toxic agents is undesirable.

Moreover, these techniques often require lengthy dialysis procedures which can never completely remove the solvent or detergent employed. See, for example, Allen, et al., Biochimica et Biophysica Acta, 601:328 (1980). Further, a variety of protocols are required depending on the lipid species. For example, the limited solubility of certain lipids (e.g., cholesterol,

-5-

phosphatidylethanolamine (PE), and phosphatidylserine (PS)) in ether or ethanol requires modification of techniques employing these solvents. Alternatively, detergent dialysis procedures employing non-ionic detergents such as octylglucoside are tedious to
5 apply as they can involve several days of dialysis. Plainly, the need to separate lipid solubilizing agents from the finished liposomes materially decreases the usefulness of these methods.

Along these same lines, the prior art LUV techniques have, in general, produced liposomes of various sizes, as well as aggregates of liposomes, thus requiring the additional step of sizing
10 the finished liposomes with a series of filters. Again, this makes the overall process more time consuming and complicated.

The fusion techniques include similar drawbacks. For example, the calcium ion/calcium chelating agent technique, like the
15 solvent and detergent techniques, involves the use and subsequent removal of materials in addition to those actually making up the finished liposomes, in this case, the chelating agent and the added calcium ions. As with the solvents and detergents, these materials represent possible sources of contamination, limit the usefulness of the technique, and make the technique more complicated.
20 Also, this technique requires that the composition of the liposomes includes some phosphatidylserine.

As to the freeze-thaw technique, this technique suffers from the drawback that the specific trapping capacity of the liposomes
25 produced by the technique drops off sharply at phospholipid concentrations above about 20 mg/ml.

The SUV techniques have similar problems. For example, high energy sonication can cause oxidation and degradation of phospholipids and may damage solute molecules which one wants to
30 capture in the interior space of the liposomes. Also, when performed using a sonication probe, high energy sonication can cause probe erosion, and if done with bath sonication in combination with radioactive materials, can produce a potentially hazardous aerosol. Low energy sonication is slow, can be destructive to
35 phospholipid molecules, and cannot be used to prepare large

-6-

quantities of liposomes. Further, the sonication approach results in low trapping efficiencies.

The infusion type SUV procedures suffer the same problems as the LUV infusion procedures. The high pressure French press technique has its own set of problems, including difficulties in making the technique repeatable, the need for post-preparation filtration to remove those MLVs which have not been converted to SUVs, the need for expensive and cumbersome equipment capable of withstanding the high pressures used, and contamination of the product by disintegration of components of the apparatus which occurs during processing of the liposomes. See, for example, Bosworth, et al., Journal of Pharmaceutical Sciences, 71:806 (1982). Also, this technique can only produce small liposomes having a low trapping efficiency.

Turning to the size distribution aspects of the invention, various procedures have been investigated in the past in an attempt to find a way to control both liposome size and distribution. Each of these procedures has fallen short of the mark in one way or another. For example, Ching-hsien Huang, in Biochemistry, 8:344 (1969), described a multi-step technique for producing a homogeneous population of small unilamellar liposomes (SUVs) which involved sonicating a lipid suspension in a buffer for 2½ hours, centrifuging the resulting product at 105,000 x g for 1 hour to remove undispersed lipid, filtering the supernatant through an extensively washed 0.1 micron Sartorius filter, subjecting the filtrate to molecular sieve chromatography on a Sepharose 4B column which had previously been saturated with the lipid suspension and washed and equilibrated with the buffer, and collecting the second fraction eluted from the column. Although this procedure did produce a population of liposomes having a defined size distribution, it was obviously complicated and time consuming to use, it only produced SUVs, and it ran the risk of chemically changing the liposomes or their contents during either the long term sonication or the exposure to the Sepharose 4B column.

-7-

In an attempt to overcome some of the problems with the Huang technique, Barenholz, et al., Biochemistry, 16:2806 (1977), developed a technique in which high speed centrifugation was substituted for molecular-sieve chromatography. In accordance with this technique, a lipid dispersion in a buffer was sonicated for 30 minutes, centrifuged for 15-30 minutes at 100,000 x g to remove large multilamellar liposomes and sonication probe particles, and the supernatant from the 100,000 x g centrifugation was re-centrifuged at 159,000 x g for periods of time ranging from 1 to 4 hours depending on the lipids, buffer compositions and temperatures used. This latter centrifugation produced three zones, the top one of which contained the desired homogeneous population of liposomes and had to be carefully removed without picking up part of the adjacent second zone. Although this technique did eliminate the use of Sepharose 4B columns, it was still long and complicated, still only produced SUVs, and still had the problems arising from sonication. Along these same lines, Watts, et al., Biochemistry, 17:1792 (1978), reported preparing a homogeneous population of SUVs from dimyristoylphosphatidylcholine (DMPC) by sonication followed by centrifugation at 105,000 x g for 10 minutes at 4°C.

In addition to the efforts directed at obtaining homogenous populations of SUVs, numerous attempts have been made to obtain homogenous populations of larger liposomes, i.e., liposomes having diameters larger than about 50 nm. The majority of these efforts have involved the use of a series of polycarbonate filters of decreasing pore size.

For example, Olson, et al., in Biochimica et Biophysica Acta, 557:9 (1979), described the sequential extrusion of large multilamellar liposomes through polycarbonate filters having pore sizes of 1.0, 0.8, 0.6, 0.4, and 0.2 microns. See also Brendzel, et al., Biochimica et Biophysica Acta, 596:129 (1980). Olson's laboratory also reported the application of their technique to the sizing of large unilamellar liposomes prepared by reverse phase evaporation. See Biochimica et Biophysica Acta, 601:559 (1980).

-8-

In this case, filters having pore sizes of 0.4, 0.2, 0.1, and 0.08 microns were used.

Although the Olson work as reported in the literature would appear to produce unimodal populations of large liposomes (see, for example, Figures 1e and 3d in the 1979 article, and Figures 1D, 2D, and 3D in the 1980 article), as described in detail in Example 10, infra, it has been surprisingly found that when quasi-elastic light scattering is used as the technique for determining size distributions, liposomes prepared by the Olson technique turn out not to have a unimodal distribution. In terms of large scale commercial production of liposomes, quasi-elastic light scattering is at present the only known real-time physical method for defining size distributions, so that in terms of commercial applications, the Olson procedure cannot be said to actually produce a population of liposomes which is unimodal.

In addition to the Olson sequential polycarbonate filter approach, other techniques have been tried in the hope of obtaining a homogenous population of relatively large liposomes. For example, Schullery, et al., Chemistry and Physics of Lipids, 12:75 (1973), described the use of Millipore filters having pore sizes of 8.0, 1.2, 0.80, 0.65, and 0.45 microns to size large multilamellar phosphatidylcholine liposomes.

Rhoden, et al., Biochemistry, 18:4173 (1979), reported the production of liposomes having diameters between 34 and 128 nm by solubilizing phosphatidylcholine and cholesterol in a sodium cholate solution and then removing that detergent by hollow fiber dialysis. The size of the liposomes was varied by adjusting the phospholipid/cholesterol ratios and the pH and ionic strength of the dialysate. It was observed that broader distributions were produced for larger liposomes.

Bosworth, et al., Journal of Pharmaceutical Sciences, 71:806 (1982), combined the sequential polycarbonate filter sizing technique with dialysis across the same types of filters. Liposomes were prepared by mechanical agitation or by the French press technique of Barenholz, et al., FEBS Lett., 99:210 (1979). Those produced by mechanical agitation were sized using filters having

-9-

pore sizes of between 0.2 and 1.0 microns, with the liposomes being passed twice through the smallest filter and, in some cases, twice through each of the filters. For dialysis, filters having pore sizes between 0.05 and 3 microns were used.

5 Enoch, et al., Proc. Natl. Acad. Sci. USA, 76:145 (1979), described the preparation of liposomes having diameters of 100 nm by detergent treatment of sonicated vesicles followed by gel filtration on Sepharose 4B. Hamilton, et al., Journal of Lipid Research, 21:981 (1980), described the preparation of liposome populations of various sizes using a French press in combination with
10 ultracentrifugation and gel chromatography on columns of 2% or 4% agarose. Reeves, et al., J. Cell. Physiol., 73:49 (1969), reported the production of a population of giant liposomes (mode = 1,200 nm) having a log-normal distribution, but vesicles smaller than
15 1000 nm were measured with difficulty, and those smaller than 500 nm were not measured at all.

A review of some of the foregoing procedures can be found in Szoka, et al., Ann. Rev. Biophys. Bioengr., 9:467, 493-494 (1980). See also Liposomes, Marc J. Ostro, ed., Marcel Dekker, Inc., New
20 York, 1983, Chapter 1.

SUMMARY OF THE INVENTION

In view of the foregoing state of the art, it is evident that there is a substantial and continuing need for an improved method for preparing unilamellar liposomes of both the SUV and LUV types.
25 Moreover, it is also evident that since at least as early as 1969, there has been a continuing effort to produce populations of liposomes having defined size distributions. Much of that effort has been directed towards obtaining populations having mean diameters greater than about 50 nm. Along with the desire for the populations per se, there has been a parallel desire for a generally
30 applicable and simple to use technique which will reproducibly generate populations of the type desired for a wide variety of processing conditions.

Accordingly, it is one of the objects of the present invention to provide an improved technique for producing unilamellar
35 liposomes. More particularly, it is an object of this invention

-10-

to provide a simple, reproducible technique for producing unilamellar liposomes which can be performed with readily available and relatively inexpensive equipment, which has a minimum number of steps, which has a high output of liposomes per unit time, and which does not require that the components making up the liposomes be sonicated or combined with solvents, detergents or other extraneous materials.

It is another object of the invention to provide a technique for producing liposomes of both the unilamellar and multilamellar types which does not require the use of solvents, detergents or other extraneous materials.

It is a further object of the invention to provide populations of liposomes having defined size distributions. It is an additional object of the invention to provide a straightforward method for obtaining such populations.

To achieve the foregoing and other objects, the invention, in accordance with certain of its aspects, provides a method for producing a population of substantially unilamellar liposomes which involves repeated extrusions at moderate pressures of previously formed liposomes through a filter having a pore size below a critical upper limit, specifically, below approximately 100 nm.

In this manner, the invention provides a variety of advantages over previously known systems for producing unilamellar liposomes, including the following: 1) the ability to form unilamellar vesicles from a wide range of lipids; 2) the ability to use high lipid concentrations (e.g., on the order of 300 $\mu\text{mol/ml}$) so as to easily achieve high trapping efficiencies; 3) the ability to provide reproducible and very rapid production of unilamellar vesicles, and, in particular, large unilamellar vesicles, through the use of high extrusion flow rates and automatic or semi-automatic recycling of the liposomes through the filter; 4) the ability to produce liposomes of a desired size by using a single pore size filter with minimum filter clogging problems; 5) the ability to avoid the use of organic solvents and detergents; and 6) the ability to provide an overall relatively gentle process.

-11-

In some cases, rather than completely transforming a population of multilamellar liposomes into a population of substantially unilamellar liposomes, it is desirable to only partially decrease the lamellarity of the population without reaching the fully unilamellar stage. Filters having a pore size of about 100 nm are still used in accordance with this aspect of the invention, but with a reduced number of passes through the filter.

In accordance with some of its other aspects, the invention provides a method for producing liposomes directly from a lipid powder or pellet by simply combining the powder or pellet with an aqueous buffer and then applying sufficient pressure to the lipid/buffer mixture to repeatedly pass it through a filter. If the filter has a pore size less than about 100 nm, substantially unilamellar liposomes are produced. If the filter has a pore size significantly above 100 nm, e.g., on the order of 200 nm, multilamellar liposomes are produced. Significantly, in either case, the liposomes are completely solvent free, in that, not even chloroform, as has been used in the past to produce MLVs, is required for liposome production in accordance with these aspects of the present invention.

In accordance with further of its aspects, the invention provides populations of liposomes having essentially unimodal distributions about mean diameters which are greater than 50 nm.

In accordance with still further of its aspects, the invention provides a method for increasing the homogeneity of a population of liposomes by repeatedly passing the liposomes through one or more filters of a constant pore size until the size distribution of the population becomes essentially unimodal.

The attainment of the foregoing and other objects and advantages of the present invention is described fully below in connection with the description of the preferred embodiments of the invention.

BRIEF DESCRIPTION OF THE DRAWINGS

Figures 1A and 1B are schematic diagrams of apparatus suitable for practicing the present invention. In Figure 1A, the

-12-

liposome suspension is recycled through the filter by hand, while in Figure 1B, the recycling has been partially automated.

Figure 2 shows the ^{31}P NMR signal intensity arising from egg phosphatidylcholine (EPC) multilamellar vesicles (in the presence of 5 mM MnCl_2) as a function of the number of extrusions through polycarbonate filters with 100 nm (circles) and 200 nm (squares) pore sizes. The error bars represent standard deviations ($n = 6$ for the point at 10 extrusions through the 100 nm filter; $n = 3$ for the point at 30 extrusions). All other experimental points represent the average obtained from two separate experiments. The lipid concentration in all cases was between 30 and 60 $\mu\text{mol/ml}$.

Figure 3 shows four freeze-fracture micrographs of vesicles prepared by repeated extrusion of multilamellar vesicles of varying lipid composition through polycarbonate filters: (a) soya phosphatidylcholine (PC) MLVs extruded through a 100 nm filter; (b) soya PC - soya PS (1:1) MLVs extruded through a 100 nm filter; (c) soya PE - soya PS (1:1) MLVs extruded through a 100 nm filter; (d) soya PC MLVs extruded through a filter with a 200 nm pore size. The arrow in part (d) indicates a cross fracture revealing inner lamellae. All micrographs have the same magnification and the direction of shadowing is indicated by the arrowhead in the bottom right corner of each section. In each case, the extrusion procedure was repeated 10 times on lipid systems containing 40-70 $\mu\text{mol/ml}$ phospholipid.

Figure 4 shows the size distribution of soya PC vesicles obtained after 10 extrusions through a polycarbonate filter with a 100 nm pore size. The vesicle diameters were measured from freeze fracture micrographs employing the technique of van Venetie *et al*, (1980) *J. Micros.*, 118:401-408. The black and half-tone columns represent vesicles that did and did not undergo freeze-thaw cycling, respectively.

Figure 5 shows the calorimetric behavior of hydrated dipalmitoylphosphatidylcholine (DPPC) in large multilamellar vesicles (MLVs) and in large unilamellar vesicles prepared by the extrusion technique of the present invention (LUVETs). The MLVs were formed by vortexing a dry lipid film in the bottom of a test tube in the

-13-

presence of a NaCl buffer at 50°C, whereas the LUVETs were formed by repetitive extrusion (10 times) of the MLVs (50 mg lipid/ml) through 100 nm pore size polycarbonate filters at 50°C. Scan rates of 2.0 °K/min were employed.

5 Figure 6 shows the trapping efficiency as a function of lipid concentration for liposomes prepared in accordance with the present invention both with (open circles) and without (closed circles) freeze-thawing. ¹⁴C-inulin was used as a trap marker.

10 Figure 7 shows the ³¹P NMR signal intensity arising from egg phosphatidylcholine (EPC) multilamellar vesicles (in the presence of 5 mM MnCl₂) as a function of the number of extrusions through polycarbonate filters with 50 nm (open circles) and 30 nm (solid circles) pore sizes. The lipid concentration in all cases was 100 mg/ml.

15 Figures 8 and 9 show freeze-fracture micrographs of the vesicles of Figure 7 processed through 50 nm and 30 nm filters, respectively. In each case, the upper portion of the figure (Figures 8A and 9A) was prepared after one extrusion (x1) through two stacked polycarbonate filters, and the lower portion (Figures 8B and 9B) after ten extrusions (x10).

20 Figure 10 shows the clearance of ¹²⁵I-tyraminyl-inulin (¹²⁵ITI) entrapped in egg phosphatidylcholine (PC) - cholesterol (1:1) LUVETs from the rat circulation (circles) and subsequent excretion in the urine (squares). The LUVETs were prepared in accordance with the present invention and were injected into the tail vein of 150-175 g female Wistar rats at a dose level of 0.5 umol phospholipid in 100 ul HBS. Urine was collected in metabolic cages. Blood was withdrawn and the animals sacrificed at the indicated times and the total amount of ¹²⁵ITI in the blood calculated assuming 4.9 ml blood per 100 g rat. Results are expressed as percentages of the total ¹²⁵ITI injected ±s.e. (n=4).

25 Figure 11 shows the long term tissue distribution of the LUVETs of Figure 10. The symbols correspond to liver (circles); carcass (triangles) and spleen (squares). Results are expressed as percentages of total ¹²⁵ITI in vivo (total ¹²⁵ITI injected minus amount excreted) ±s.e. (n=4).

-14-

DESCRIPTION OF THE PREFERRED EMBODIMENTS

As described above, the present invention provides extrusion techniques for producing: 1) populations of substantially unilamellar liposomes; and 2) populations of liposomes having substantially unimodal distributions (hereinafter referred to as the "unilamellar" and "unimodal" aspects of the invention, respectively). In addition, the invention allows liposomes to be produced without the use of any solvents, detergents or other extraneous materials (hereinafter referred to as the "solvent free" aspects of the invention).

The populations of substantially unilamellar liposomes are produced by subjecting previously formed liposomes to multiple extrusions at moderate pressures through a filter having a pore size of less than or equal to about 100 nm.

The previously formed liposomes can have a variety of compositions and can be prepared by any of the techniques now known or subsequently developed for preparing liposomes.

For example, the previously formed liposomes can be formed by the conventional technique for preparing MLVs, that is, by depositing one or more selected lipids on the inside walls of a suitable vessel by dissolving the lipids in chloroform and then evaporating the chloroform, adding the aqueous solution to be encapsulated to the vessel, allowing the aqueous solution to hydrate the lipid, and swirling or vortexing the resulting lipid suspension to produce the desired liposomes. This technique employs the most gentle conditions and the simplest equipment and procedures known in the art for producing liposomes. Also, this technique specifically avoids the problems with sonication or the use of detergents, solvents (other than chloroform) or other extraneous materials, discussed above.

Alternatively, in accordance with the solvent free aspects of the present invention, the liposomes which are to be repeatedly extruded through the filter can be prepared by simply mixing a lipid powder or pellet and buffer together and then directly extruding that mixture through the filter. If the filter has a pore size of less than about 100 nm, this procedure produces

-15-

unilamellar liposomes, while if the pore size is substantially greater than 100 nm, multilamellar liposomes are produced. In either case, the procedure eliminates the use of all solvents, including chloroform.

5 With regard to the production of populations of liposomes having substantially unimodal distributions, the liposomes making up the population can have a variety of compositions and can be in the form of multilamellar, unilamellar, or other types of liposomes or, more generally, lipid-containing particles, now
10 known or later developed. For example, the lipid-containing particles can be in the form of steroidal liposomes, stable plurilamellar liposomes (SPLVs), monophasic vesicles (MPVs), or lipid matrix carriers (LMCs) of the types disclosed in copending and commonly assigned U.S. Patent Applications Serial Nos.
15 476,496, 521,176, 591,576 and 599,691, filed March 24, 1983, August 8, 1983, March 20, 1984, and April 12, 1984, respectively, the pertinent portions of which are incorporated herein by reference.

 The mean diameter of the population will depend on the manner
20 in which the liposomes are to be used. For example, as recognized by persons skilled in the art, for diagnostic applications, mean diameters in the range of 100 nm to 500 nm are generally preferred, while for depoting of drugs, larger diameters, e.g., on the order of 500 nm to 1000 nm, are preferred, and for applica-
25 tions where endocytosis is desirable, smaller diameters, e.g., on the order of 50 nm to 100 nm, are preferred. Similar ranges have been recognized in the art for other applications. See, for example, Liposomes, supra.

 Mean diameters of populations of liposomes can be measured by
30 various techniques known in the art, including freeze-fracture and quasi-elastic light scattering. As discussed above and in more detail below, quasi-elastic light scattering is preferred in the context of the unimodal aspects of the present invention, and the values for liposome diameters reported herein in connection with
35 those aspects were measured using this technique.

 The substantially unimodal population of liposomes is prepared by repeatedly passing previously formed liposomes through

-16-

filters of a constant pore size until the size distribution of the population in fact becomes unimodal. It has been surprisingly found that repeated passages through filters of a constant pore size causes bimodal aspects of the original size distribution of the liposomes, as well as any higher modal aspects, to eventually disappear. For the method to work, however, it is necessary to use one pore size, and use it repeatedly.

The previously formed liposomes can be prepared by any of the techniques now known or subsequently developed for preparing liposomes. For example, the previously formed liposomes can be formed by the conventional technique, discussed above, for preparing multilamellar liposomes (MLVs).

Alternatively, techniques used for producing large unilamellar liposomes (LUVs), such as, reverse-phase evaporation, infusion procedures, and detergent dilution, can be used to produce the previously formed liposomes. A review of these and other methods for producing liposomes can be found in the text Liposomes, supra, the pertinent portions of which are incorporated herein by reference. As other alternatives, the previously formed liposomes can be produced in accordance with the procedures described in U.S. Patent Applications Serial Nos. 476,496, 521,176 and 599,691, referred to above. Also, rather than using liposomes per se, other lipid-containing particles, such as those described in U.S. Patent Application Serial No. 591,576, referred to above, can be used in the practice of the present invention. In such cases, the resulting unimodal population will, in general, not be a population of liposomes, but rather a population having similar characteristics to those of the original lipid-containing particles used.

In choosing a technique for producing the previously formed liposomes, it is important to select one that will not produce a substantial number of liposomes having a diameter significantly smaller than the pore size selected for generating the unimodal population. Otherwise, it may take an extremely high number of passes through the filter to incorporate the small liposomes into the unimodal population. Since size distributions for populations

-17-

of liposomes are relatively easily determined, selecting a technique which satisfies this requirement is well within the skill of persons skilled in the art.

5 Rather than using previously formed liposomes as the starting material, if desired, the substantially unimodal population of liposomes can be prepared using the solvent free approach, discussed above. That is, the population of liposomes can be prepared directly from a lipid powder or pellet and buffer by simply mixing these ingredients together and then directly passing that
10 mixture through filters of a selected pore size a sufficient number of times to achieve the desired unimodality.

The filter used to generate the unilamellar liposomes or the unimodal distribution of liposomes is preferably of the type which has straight through channels. Polycarbonate filters of this type
15 produced by Nuclepore, Inc., Pleasanton, CA, have been found to work successfully in the practice of the present invention. In a typical procedure, the filter may have to be changed after the first two or three passes of the liposome suspension due to pore clogging. Clogging in general depends on such variables as lipid
20 composition, purity and concentration, as well as on the pressure and flow rates used.

The most critical parameter in preparing unilamellar liposomes in accordance with the present invention is the size of the filter's channels. It has been found that unilamellar
25 liposomes cannot be produced from multilamellar liposomes, no matter how many times the MLVs are passed through the filter, if the filter's pore size is significantly above about 100 nm, e.g., if the pore size is about 200 nm (see Example 2, infra). Accordingly, only filters having a pore size equal to or below about 100 nm
30 can be used in connection with this aspect of the invention.

As illustrated in Example 6 below, the size of the unilamellar liposomes produced depends on the pore size of the filter used, the mean diameter being, in general, somewhat smaller than the pore size. If desired, the liposome's mean diameter, as
35 well as their trapped volumes (ul per umol phospholipid), can be easily increased using the freeze-thaw procedure discussed above

-18-

(see also Example 4, infra). Importantly, since this procedure does not involve the use of solvents, detergents or other extraneous materials, the increase in liposome size is not at the expense of introducing contamination and degradation problems. Vesicle size and trapped volumes can also be manipulated by varying other parameters of the system, such as, lipid composition.

The number of passes through the filter needed to produce the desired unilamellar liposomes depends on the filter characteristics (pore size, composition and geometry) and the materials from which the liposomes are to be made. As illustrated by Example 2, infra, five or more passes through a double stacked polycarbonate filter having a pore size of 100 nm are typically required to obtain unilamellar liposomes. Less passes may be needed for smaller pore sizes. For example, with 30 nm and 50 nm filters, two to four passes are in general sufficient to produce a substantially unilamellar population of liposomes (see Example 6, infra). Also, if the goal is only to reduce the lamellarity of the population, rather than to achieve substantial unilamellarity, less passes are needed. The appropriate number of passes for any particular system can easily be determined by persons of ordinary skill in the art by simply sampling the finished liposomes to determine when the desired degree of lamellarity has been achieved.

With regard to the unimodal aspects of the invention, the pore size of the filter is the primary determinant of the mean diameter of the final population. In general, within approximately 15 to 50 percent, the mean diameter is approximately equal to the pore size. However, for pore sizes below about 100 nm, the mean diameter of the population tends to level off at about 75 nm as measured by quasi-elastic light scattering, irrespective of the specific pore size used. As discussed above, for pore sizes below about 100 nm, the finished liposomes are found to be substantially unilamellar, irrespective of the lamellarity of the original population. For pore sizes above 100 nm, multilamellar liposomes remain multilamellar and unilamellar liposomes remain unilamellar.

The number of passes through the filter needed to produce a substantially unimodal population depends on the filter

-19-

characteristics (pore size, composition and geometry) and the materials from which the liposomes are to be made. In some case three to five passes through double stacked polycarbonate filters are sufficient to produce a unimodal population. As a general proposition, 25 passes through double stacked polycarbonate filters will produce the desired unimodal distribution for most liposome preparations. The appropriate number of passes for any particular system can easily be determined by persons of ordinary skill in the art by simply sampling the finished liposomes to determine when substantial unimodality has been achieved.

Passage of the liposomes through the filter to produce the unimodal, unilamellar, or both unimodal and unilamellar population of liposomes is accomplished under pressure. Pressures of various magnitudes can be used depending upon the type and composition of the liposomes to be produced, the specific characteristics of the equipment employed, and the rate at which liposomes are to be produced.

Maximum pressures generally are limited by the pore size of the support used to hold the filter. For a filter support having a pore size of about 30 microns, pressures between about 100 and 700 psi have been found to work successfully. These pressures produce intact liposomes, give high flow rates (on the order of 20-60 ml/min for a double stacked polycarbonate filter having a pore size of 100 nm), and produce homogenous size distributions, e.g., 60-100 nm diameter liposomes for a 100 nm filter. With a filter support having a pore size smaller than 30 microns, higher pressures can be used.

As with the number of passages through the filter, the appropriate pressure for a particular system can be readily determined by persons skilled in the art by examining the finished liposomes to determine if they are substantially intact and have the desired unimodal distribution and/or unilamellarity.

Pressures in the 100-700 psi range are also preferred because they allow for the extrusion of solutions having lipid concentrations on the order of about 300 umols phospholipid per ml without significant filter clogging. Prior art liposome sizing

-20-

techniques employing polycarbonate filters used pressures less than 100 psi, and thus were limited to lipid concentrations of 60 umol/ml. The use of high lipid concentrations has resulted in trapping efficiencies on the order of 30% for the present invention. Rapid extrusion rates on the order of 20 ml/min and above are still achieved for such high lipid concentrations when pressures in the range of 300-500 psi are used.

Flow rates on the order of 20-60 ml/min do not represent the maximum flow rates that are achievable with the present invention, but merely represent rates consistent with convenient collection of the extruded material. Maximum flow rates are sensitive to the concentration of lipid, the history of the sample (i.e., whether it has been extruded one or more times), the pressure employed, and the nature of the lipids themselves. For example, "gel state" lipids cannot be extruded. Such lipids (e.g., dipalmitoyl phosphatidylcholine (DPPC)) must be heated above their gel to liquid crystalline transition temperature (41°C for DPPC) before the extrusion process can proceed.

At increased pressure, the extrusion can be exceedingly rapid. For example, a 5 ml dispersion of 50 mg/ml egg phosphatidylcholine (EPC) has been extruded through a 100 nm pore size filter in less than 2 seconds at 500 psi, corresponding to a flow rate of at least 150 ml/min. This rate would be increased further by higher pressure or temperature.

Suitable apparatus for practicing the present invention is shown in Figures 1A and 1B. As shown in Figure 1A, liposome suspension 4 which is to be extruded through filter 6 is introduced into pressure chamber 2 by means of injection port 8. The injection port also serves as a release valve. Pressure chamber 2 is formed from upper portion 10 and lower portion 12 which are connected together by, for example, bolts 14. A seal between these portions and filter 6 is provided by O-ring 16. Preferably, the chamber is made of clear plastic so that the extrusion of the suspension from the chamber can be visually observed. The filter is supported within chamber 2 by filter support 18. In practice,

-21-

it has been found convenient to use two stacked polycarbonate filters to form filter 6.

Pressure is supplied to chamber 2 by means of conduit 20 which is connected to a source of pressure, e.g., a high pressure nitrogen tank (not shown). Conduit 20 includes valve and regulator 22 for adjusting the pressure within chamber 2. The material extruded from filter 6 is removed from chamber 2 by means of conduit 24 and collected in receiving vessel 26. In practice, valve 22 is closed prior to the time all of the suspension has been extruded from chamber 2 so as to prevent high pressure gas from flowing through the system and blowing the suspension out of receiving vessel 26. After having been collected in vessel 26, the extruded material is repeatedly returned to chamber 2 by means of injection port 8, until the original population of liposomes has passed through filter 6 a sufficient number of times so as to substantially increase its unimodality and/or unilamellarity.

Figure 1B shows a modification of the apparatus of Figure 1A wherein the recycling of the extrudate is partially automated. The apparatus shown in this figure is used as follows.

First, a filter 6 is installed in the apparatus by removing threaded retainer plug 5, filter support housing 9, and O-ring 16 from aluminum housing 7. A filter is then placed on the filter support and the components reassembled with plug 5 being tightened until O-ring 16 is compressed against inner plexiglas housing 11 contained within outer aluminum housing 13. If desired, a porous drain disc (not shown) can be placed under the filter.

A sample is then loaded into receiving chamber 3 by rotating load/recycle/discharge valve 15 until load/discharge tube 17 is aligned with inlet port 19, and by rotating pressure/vent valve 23 until vent port 25 is aligned with exhaust port 21. A sample can then be introduced into the receiving chamber through load/discharge tube 17. Most conveniently, this is done by attaching a short length of flexible tubing and a hypodermic syringe to load/discharge tube 17.

Once the sample has been completely loaded into receiving chamber 3, it is transferred to pressure chamber 2 by depressing

-22-

transfer valve 27. The sample is now ready for extrusion through filter 6. To perform the extrusion, pressure/vent valve 23 is rotated until gas inlet port 31 is aligned with pressure port 33, and load/recycle/discharge valve 15 is rotated to a position where recycle port 37, formed in valve 15, is aligned at one end with inlet port 19 and at the other end with bypass port 29. Gas inlet port 31 is connected to threaded aperture 35 which serves to connect the apparatus to a valved and regulated external source of high pressure gas, e.g., a valved and regulated high pressure nitrogen tank (not shown).

Pressure is then applied to pressure chamber 2 causing the sample to pass from that chamber to receiving chamber 3 by way of filter 6, bypass port 29, recycle port 37, and inlet port 19. This accomplishes one extrusion of the sample through the filter. Flow from pressure chamber 2 to receiving chamber 3 can be visually observed through plexiglas housing 11, and the amount of pressure applied can be adjusted to achieve a gentle flow.

Once all of the sample has been transferred to receiving chamber 3, the valve on the external source of pressure is closed, and pressure/vent valve 23 is rotated to first bring vent port 25 into alignment with exhaust port 21 and then into alignment with pressure port 33, thus venting both receiving chamber 3 and pressure chamber 2. The sample can now be reintroduced to pressure chamber 2 by simply depressing transfer valve 27. With the sample in pressure chamber 2, the extrusion process is repeated following the procedures described above for the first extrusion. Note that with the sample in the receiving chamber and with both chambers having been vented, a new filter can be installed, if desired, following the procedures described above.

Once the receiving chamber - pressure chamber - receiving chamber cycle has been repeated the desired number of times, the sample is discharged from the apparatus through load/discharge tube 17 by first aligning gas inlet port 31 with exhaust port 21 and load/discharge tube 17 with inlet port 19, and then applying pressure to the system from the external pressure source. In practice, the external pressure is shut off before all of the

-23-

sample has left chamber 3 to avoid high gas flows at the end of the evacuation. Rather than using load/discharge tube 17, the sample can also be removed by disassembling threaded retainer plug 5, filter support housing 9, and O-ring 16 from aluminum housing 7, and then depressing transfer valve 27 to cause the sample to flow into the pressure chamber and out the bottom of the apparatus.

If desired, the apparatus shown in Figure 1B, or equivalent apparatus, can be equipped with conventional automatic fluid handling equipment and controls to achieve a completely automated process. Also, so as to be able to maintain the temperature of the sample above the gel to liquid crystalline transition temperature of the lipids used, the pressure chambers of whatever equipment is employed can be heated using a water jacket or similar device.

Without intending to limit it in any manner, the present invention will be more fully described by the following examples. The materials and methods which are common to the various examples presented below are as follows.

Materials and Methods

Lipids

Egg phosphatidylcholine (EPC) and soya phosphatidylcholine (SPC) were isolated employing standard procedures (Singleton, et al., Journal of the American Oil Chemical Society, 42:53 (1965)). Corresponding varieties of phosphatidylethanolamine (PE) and phosphatidylserine (PS) were prepared from EPC and SPC to produce EPE, SPE, EPS and SPS (see Comfurius, P. and Zwaal, R.F.A. (1977) Biochim. Biophys. Acta, 488:36-42). The lipids from the soya source are considerably more unsaturated than those derived from EPC, due to the high content of linoleic acid in SPC (see Tilcock, C.P.S. and Cullis, P.R. (1981) Biochim. Biophys. Acta, 641:189-201). All lipids were more than 99% pure as determined by TLC. Acidic phospholipids (PS) were converted to the sodium salt form as described in Hope, M.J. and Cullis, P.R. (1980) Biochem. Biophys. Res. Commun., 92:846-852. Cholesterol (Sigma, St. Louis) was used without further purification.

-24-

Determination of Trapped Volumes

To determine trapped volumes, multilamellar vesicles were prepared in accordance with the procedures described in Example 1, infra, but in the presence of 1 uCi of ^{22}Na or ^{14}C -inulin (NEN, Canada). Unilamellar liposomes were then prepared from the multilamellar liposomes, again following the procedures of Example 1.

An aliquot (100 ul) was then loaded onto a Sephadex G-50 column packed in a 1 ml disposable syringe, and vesicles eluted by centrifugation of the column at 500 g for 3 min. See Pick, U. (1981) Arch. Biochem. Biophys., 212:186-194. In the case of ^{22}Na this was sufficient to remove all the untrapped material. However, to remove all the untrapped inulin this procedure was either repeated once more or a single pass through an Ultragel column (LKB - ACA34) was employed. Aliquots of the eluted material were assayed for lipid phosphorus according to the method of Bottcher, C.J.F., Van Gent, C.M. and Pries, C. (1961) Anal. Chim. Acta, 24:203-204; trapped ^{22}Na was determined employing a Beckman 8000 gamma counter and trapped ^{14}C inulin was estimated using a Phillips PW-4700 liquid scintillation counter. Trapped volumes were then calculated as ul of trapped volume per umol of phospholipid.

^{31}P Nuclear Magnetic Resonance

^{31}P NMR was employed to provide an indication of the extent to which the vesicle preparations were unilamellar. Specifically, Mn^{2+} was added to the vesicle dispersion (3 ml, 30-60 umol phospholipid per ml in a 15 mm diameter NMR tube) at levels (5 mM) sufficient to broaden beyond detection the ^{31}P NMR signal from those phospholipids facing the external medium. If the vesicles are large and unilamellar, then approximately 50% of the signal should remain following the addition of Mn^{2+} . The impermeability of the vesicles to Mn^{2+} was demonstrated by following the timecourse of the signal intensity, which for the PC systems investigated was found to be stable over a period of days. Spectra were obtained employing a Bruker WP 200 NMR spectrometer operating at 81 MHz. Accumulated free induction decays corresponding to

-25-

1000 transients were collected using a 15 usec 90° radiofrequency pulse, gated proton decoupling and a 20 KHz sweep width. An exponential multiplication corresponding to a 50 Hz linebroadening was applied prior to Fourier transformation. Signal intensities were measured by cutting out and weighing spectra with triphenylphosphite (in a small central capillary in the NMR tube) as a reference.

Determination of Vesicle Size Distributions

Vesicle size distributions for Examples 1-7 and part of Example 8 were determined by freeze-fracture. Vesicle preparations were mixed with glycerol (25% by volume) and frozen in a freon slush. Samples were fractured and replicated employing a Balzers BAF 400D apparatus, and micrographs of replicas were obtained using a Phillips 400 electron microscope. Vesicle size distributions were determined by measuring the diameter of fractured vesicles that were 50% shadowed according to the procedure of van Venetie et al., (1980) J. Micros., 118:401-408.

Vesicle size distributions for part of Example 8 and Examples 9-10 were determined using quasi-elastic light scattering, also known as dynamic light scattering or photon-correlation spectroscopy.

As known in the art, this technique involves passing coherent light, e.g., light produced by a helium-neon laser, through a sample of the suspended vesicles and measuring the time dependent fluctuations in the intensity of the light scattered by the vesicles. An autocorrelation function is then calculated from this data. As can be shown theoretically, this autocorrelation function is directly related to the diffusion coefficients of the vesicles in the sample, which in turn are a function of the hydrodynamic radii of the vesicles. Accordingly, different vesicle size distributions will produce different autocorrelation functions.

In practice, unique particle size distributions are not obtained directly from autocorrelation functions. Rather, a distribution is assumed for the vesicles, and a determination is then made as to how well the autocorrelation function calculated from the measured data fits the autocorrelation function that would be

-26-

produced if the vesicles in the sample actually had the assumed distribution.

Specifically, if it is assumed that the intensity-weighted distribution of the diffusion coefficients of the vesicles is a unimodal Gaussian distribution, then it can be shown theoretically that a second order polynomial, i.e., a polynomial in powers of t up to t^2 , will exactly fit the natural logarithm of the autocorrelation function. See D.E. Koppel, Journal of Chemical Physics, 57:4814 (1972). Accordingly, the extent to which a second order polynomial, i.e., a quadratic, in fact does fit the natural logarithm of the autocorrelation function for a particular sample is an accurate measure of the extent to which the diffusion coefficients of the vesicles in the sample have a unimodal Gaussian distribution. As used in the art, this procedure is often referred to as a cumulants analysis of the autocorrelation function.

As known in the art, it is a straightforward matter to 1) determine the natural logarithm of an autocorrelation function, 2) to fit a second order polynomial to the natural logarithm, and 3) to determine the goodness of fit of that polynomial to that logarithm. Accordingly, the Gaussian distribution approach is at present the most practical way to characterize and compare populations of vesicles, and thus it is the approach used herein in connection with the unimodal aspects of the present invention.

Specifically, in accordance with those aspects, a population of liposomes is considered to be substantially "unimodal" when the logarithm of its autocorrelation function fits closely to a second order polynomial. In the examples presented below, autocorrelation functions were obtained using a Nicomp Model 200 Laser Particle Sizer (Nicomp Instruments, Inc., Santa Barbara, California). This equipment uses the standard method of least-squares for curve fitting and reports goodness of fit as a χ^2 value derived from the deviations between the logarithm of the autocorrelation function and the values predicted by the second order polynomial. Values of χ^2 in the range of 0-2 indicate a good fit of the data by the assumed unimodal Gaussian distribution, while high values of χ^2 indicate a poor fit.

-27-

For a good fit, an estimate of the standard deviation of the distribution can be derived from the square root of the coefficient of the second order term of the polynomial. For distributions having a good fit, this estimate is reported in the examples, while for poor fits, the estimate is not reported, since, although a value can be calculated, such a value does not in fact serve as an estimate of the standard deviation.

Other Chemicals

Inulin, periodic acid, sodium-m-arsenite, tyramine, G-25 Sephadex, sodium cyanoborohydride, sodium borohydride, and cholesterol were obtained from Sigma. Ultrogel Ac34 was obtained from Pharmacia, carrier free Na^{125}I (100 mCi/ml) was supplied by Amersham and iodogen was obtained from Pierce. All other chemicals were of analytical grade.

Example 1

Preparation of Unilamellar Liposomes

This example illustrates the preparation of large unilamellar liposomes using the extrusion method of the present invention. For ease of reference, liposomes prepared in accordance with this technique are referred to herein by the acronym "LUVETs," i.e., Large Unilamellar Vesicles by Extrusion Techniques.

Large multilamellar vesicles (MLVs) were prepared by the conventional process as follows. First, lipid dissolved in chloroform was dried down and deposited as a film on the inside of a test tube. The MLVs were then formed by simply adding an aqueous buffer of 150 mM NaCl, 20 mM HEPES, pH 7.5, to the test tube and hydrating the lipid by vortex mixing.

The resulting MLV dispersion (2-10 ml) was then transferred into the pressure chamber of the apparatus shown in Figure 1A, equipped with two stacked standard 25 mm polycarbonate filters having a 100 nm pore size (Nuclepore, Inc., Pleasanton, California, Catalog # 110605). Nitrogen pressure was applied to the chamber via a standard gas cylinder fitted with a high pressure (0-4000 psi) regulator. The vesicles were extruded through the filter employing pressures of 100-700 psi resulting in flow rates of 20-60 ml/min, and were collected and re-injected.

-28-

Repetition of the extrusion procedure five or more times resulted in the production of large unilamellar liposomes having diameters of approximately 70 nm as measured by freeze fracture. The overall extrusion process including recycling generally took fifteen minutes or less.

The following examples describe in detail the size, unilamellarity, trapped volume, trapping efficiency and influence of various lipid compositions on liposomes produced by the foregoing procedure. Also, the effects of a freeze-thaw procedure on trapped volume and the criticality of filter pore size are illustrated.

Example 2

Criticality of Filter Pore Size

This example demonstrates the criticality of filter pore size in producing unilamellar liposomes, and, in particular, the criticality of using a filter having a pore size of less than or equal to about 100 nm.

EPC MLVs were prepared in accordance with the procedures described in Example 1 and then repeatedly passed through polycarbonate filters having pore sizes of 100 and 200 nm. The unilamellarity of the resulting liposomes was determined using the ^{31}P NMR technique described above. The results are shown in Figure 2.

As shown in that figure, for vesicles passed through the 200 nm filter, the signal intensity drops to approximately 65% after five passes through the filter and then remains relatively constant. For the 100 nm filter, on the other hand, the signal drops to approximately 50% after five or more passes.

Since a drop in signal intensity to about 50% indicates that the liposomes are substantially unilamellar, while a drop to only 65% indicates substantial multilamellarity, these results show that the 100 nm filter succeeds in producing unilamellar liposomes, as desired, while the 200 nm filter continues to produce significant amounts of multilamellar liposomes irrespective of the number of passes through the filter.

-29-

This conclusion is confirmed by the freeze-fracture micrographs shown in Figure 3. As shown in that figure, vesicles formed from SPC, SPC-SPS (1:1) and SPE-SPS (1:1) (Figures 3(a), (b) and (c), respectively) using a 100 nm filter do not exhibit a significant number of cross-fractures (less than 0.1%) indicating the absence of inner lamellae. In contrast, cross-fractures are readily observable for SPC vesicles processed through a 200 nm filter (Figure 3(d)).

These results clearly establish that in accordance with the present invention, unilamellarity depends upon the use of a filter having a pore size on the order of 100 nm or below.

Example 3

LUVET Diameters, Trapped Volumes and Unilamellarity

This example demonstrates that the procedures of the present invention when used with 100 nm filters reproducibly result in the production of a relatively homogeneous population of LUVs for a variety of lipid constituents. Vesicle diameters and trapped volumes were determined by the methods described above. The results are shown in Figure 4 and Table I, infra.

The half-tone columns in Figure 4 show the vesicle diameters measured for SPC LUVETs which were prepared by passing MLVs prepared in accordance with Example 1 through two (stacked) 100 nm pore size filters ten times. Table I shows in summary form the measured mean diameters and mean trapped volumes for this and other lipid compositions. As a control, EPC LUVs were prepared by two procedures (octylglucoside detergent dialysis and reverse phase evaporation) which are generally accepted to produce unilamellar vesicles, and the LUVs so produced were then extruded ten times through two (stacked) 100 nm pore size filters. See Mimms, L.T., Zampighi, G., Nozaki, Y., Tanford, C. and Reynolds, J.A. (1981) Biochemistry, 20:833-840 and Szoka, F. and Papahadjopoulos, D. (1980) Ann. Rev. Bioeng., 9:467-508. The results for these controls are also shown in Table I. (It is of interest to note with regard to the generality of the present invention that when the octylglucoside procedure was employed to make vesicles consisting of EPC/cholesterol (1:0.25),

-30-

multilamellar vesicles were formed, whereas with the procedure of the present invention and the same lipid constituents, substantially unilamellar vesicles were formed.)

5 The vesicle diameter distribution shown in Figure 4 can be used to determine calculated values for trapped volumes and amount of inner monolayer phospholipid by assuming 1) an area per phospholipid molecule, e.g., 0.6 nm^2 (see Schieren, H., Rudolph, S., Finkelstein, M., Coleman, P. and Weissman, G. (1978) Biochim. Biophys. Acta., 542:137-153); 2) a bilayer thickness, e.g., 4 nm
10 (see Blaurock, A.E. (1982) Biochim. Biophys. Acta., 650:167-207); and 3) that the vesicles are unilamellar. These calculated values can then be compared with the experimentally observed trapped volumes and amounts of inner monolayer phospholipid to determine the proportion of unilamellar vesicles present.

15 Following this approach for the vesicle size distribution shown in Figure 4 (half tone), it was determined that such a vesicle population (if unilamellar) would have an "inner monolayer" signal intensity (after the addition of Mn^{2+}) of approximately 43% of the original intensity and that the trapped
20 volume would be approximately 1.6 ul/umole. This is in reasonable agreement with the measured values of sequestered phospholipid (48%) and trapped volume ($1.2 \pm 0.2 \text{ ul/umol}$) in view of the number of assumptions made, and, in particular, in view of the difficulty in estimating the area per phospholipid molecule which can greatly
25 affect the trapped volume expressed as ul trapped per umole of phospholipid.

Comparing the calculated trapped volume value of 1.6 ul/umole with the experimental data shown in Table I reveals that LUVETs composed of SPC and EPC exhibit trapped volumes smaller than those
30 expected, while if a charged phospholipid species such as phosphatidylserine is present, the theoretical trapped volume is achieved.

Two possible reasons for the low trapped volumes observed for EPC and SPC LUVETs are that there are a significant number of
35 multilamellar vesicles present in the population, or that there are a greater proportion of small vesicles present than estimated

-31-

from the freeze-fracture micrographs. The freeze-fracture results suggest that the number of multilamellar vesicles is very small (less than 2%), even if it is assumed that only 5% of fractured multilamellar systems exhibit a cross-fracture (see R.G. Miller, Nature, 287:166 (1980)). On the other hand, an underestimation of the number of small vesicles is likely.

Moreover, as shown in Table I, the trapped volumes measured for EPC LUVs produced by the octylglucoside detergent dialysis procedure and the reverse phase evaporation procedure, which were subsequently extruded 10 times through a filter with a 100 nm pore size, are comparable to the trapped volumes obtained for the EPC LUVETs.

These observations, taken together, demonstrate that the great majority of vesicles produced by the extrusion technique of the present invention are unilamellar, even though the measured trapped volume in certain cases is smaller than the calculated value.

To establish that the procedures of the present invention when used with filters having a pore size of 100 nm produce LUVs, as opposed to SUVs, calorimetric studies were conducted on MLVs and LUVETs composed of 16:0/16:0 PC (dipalmitoylphosphatidylcholine -- DPPC).

SUVs composed of saturated phospholipids, such as, DPPC, are known to exhibit a reduction in the gel-liquid crystalline transition temperature (T_c) and a broadening of the transition due to their highly curved membranes. This high curvature is generally considered undesirable because it results in increased disorder in the membrane's hydrocarbon region (see Schuh, J.R., Banerjee, U., Muller, L. and Chan, S.I. (1982) Biochim. Biophys. Acta, 687:219-225).

In order to ascertain whether the LUVETs produced by the present invention are sufficiently large to avoid the problems arising from highly curved membranes, T_c values were calorimetrically measured for MLVs and LUVETs prepared in accordance with the procedures of Example 1. The results are shown in Figure 5.

-32-

As illustrated in this figure, the MLVs and LUVETs exhibit very similar values of T_c . These values are consistent with those reported in the literature. See Ladbroke, B.D. and Chapman, D. (1969) Chem. Phys. Lipids, 3:304-367. They are in direct contrast to the behavior observed for sonicated DPPC vesicles, which exhibit a broadened gel-liquid crystalline transition which occurs some 4°C below the melting temperature of the multilamellar systems. See van Dijck, P.W.M., de Kruijff, B., Aarts, P.A.M.M., Verkleij, A.J. and de Gier, J. (1978) Biochim. Biophys. Acta, 506:183-191. Accordingly, the unilamellar liposomes prepared by the procedures of the present invention using filters with a 100 nm pore size are properly classified as LUVs, rather than SUVs.

To test the structural integrity of the liposomes produced by the extrusion process of the present invention, LUVETs were prepared in accordance with the procedures of Example 1, but with a buffer having a NaCl concentration of 1 M, instead of 150 mM. After preparation, the liposomes were placed in distilled water creating a large osmotic pressure difference across the liposomes' membranes. Using arenazo III as a marker for liposome leakage, essentially no leakage was found under these severe test conditions.

Example 4

Use of Freeze-Thaw Cycles to Increase Trapped Volume

This example illustrates the use of a freeze-thaw procedure to increase the trapped volumes of the unilamellar liposomes produced by the present invention.

SPC and EPC LUVETs prepared in accordance with the procedures of Example 1 were subjected to two freeze-thaw cycles (employing liquid nitrogen) followed by extrusion through new 100 nm pore size filters. Specifically, the LUVETs were placed in a plastic vial, and the vial placed in liquid nitrogen for approximately 1 minute. The frozen LUVETs were then thawed in water at room temperature for approximately 5 minutes. The thawed solution was extruded 3 times through new 100 nm filters, after which the freeze-thaw-extrude process was repeated a second time.

-33-

Summary results for the process are given in Table I. Details of the size distribution of freeze-thawed SPC LUVETs is given in Figure 4 (solid columns).

As shown in Figure 4, the mean diameter of the SPC LUVETs increased by approximately 20 nm. The calculated trapped volume for this vesicle distribution is 2.3 ul/umole which is in excellent agreement with the measured value of 2.2 ± 0.1 ul/umol (Table I).

Even higher trapped volumes were achieved using a soya PC system wherein freeze-thawing of LUVETs prepared by extrusion (10 times) through the 100 nm pore size filters, followed by extrusion (three to four times) through 200 nm pore size filters, resulted in trapped volumes on the order of 10 ul/umol phospholipid.

Example 5

LUVET Trapping Efficiency

An important parameter of LUV preparations is their trapping efficiency. This is especially so when the agents to be trapped are either expensive, as is the case for many drugs, or have low solubilities.

In connection with the present invention, it has been found that the overall process can be made to have trapping efficiencies on the order of 30%, notwithstanding the relatively low trapped volumes of the vesicles produced, by simply increasing the lipid concentration of the solutions used to prepare the LUVETs.

This effect is demonstrated in Figure 6 where the percentage of aqueous volume trapped inside the LUVETs is plotted against lipid concentration for LUVETs prepared in accordance with the procedures of Example 1 (solid circles) and using the freeze-thaw procedure of Example 4 (open circles).

Preparation of LUVETs at lipid concentrations of 300 umoles/ml is easily accomplished, giving rise to trapping efficiencies on the order of 30% as shown in the figure. It is interesting to note that the freeze-thaw cycle only gives rise to significant increases in trapped volume per umol of lipid at lipid concentrations below 200 umol/ml. Similar observations have been reported by Pick, U. (1981) Arch. Biochem. Biophys., 212:186-194.

-34-

Example 6Use of Filters Having Pore Sizes Less Than 100 nm

5 This example illustrates the effects of using filters having pore sizes less than 100 nm on the size of the liposomes produced and on the number of passes through the filter needed to achieve substantial unilamellarity.

10 MLVs were prepared in accordance with the procedures of Example 1 using egg PC at a concentration of 100 mg/ml and using a buffer of 150 mM NaCl and 20 mM Hepes (pH 7.5). The dispersion was passed ten times through two stacked polycarbonate filters having a pore size of either 50 nm or 30 nm using the apparatus of Figure 1A. Aliquots were taken after one and after ten passes through the extrusion device and used to prepare freeze-fracture micrographs as described above. Samples (4ml, 25 mg phospholipid per ml) were also taken after various numbers of passes and analyzed by ^{31}P NMR using Mn^{2+} as described above. The results are shown in Figures 7-9.

20 As shown in Figure 7, vesicles extruded once through the 50 nm filters lost 37 percent of their ^{31}P NMR signal upon addition of Mn^{2+} , while vesicles extruded once through the 30 nm filters lost 47.5 percent. This indicates that the vesicles passed through the 50 nm filters are larger and/or more multilamellar than those passed through the 30 nm filter, a result which is confirmed by the freeze-fracture micrographs shown in Figures 8 and 9. Comparing the upper portions of those figures (Figures 8A and 9A) reveals that the liposomes which were passed once through the 50 nm filters are larger and more irregular than those which were passed once through the 30 nm filters.

25 After ten passes, the ^{31}P NMR signal intensities dropped by 53 and 56 percent for the 50 nm and 30 nm filters, respectively. This indicates that both filters were producing essentially the same size liposomes. This was confirmed by analysis of freeze-fracture micrographs which revealed that both populations had an average diameter of 44 ± 14 nm, i.e. a diameter characteristic of SUVs. As illustrated by Figures 8B and 9B, in each case, the population produced was homogeneous.

-35-

Comparing the curves of Figure 7 with the curve for the 100 nm filter in Figure 2 reveals that the ^{31}P NMR signals tend to level off faster for the filters with smaller pore sizes. Accordingly, less passes through the extrusion apparatus are required to achieve a population of substantially unilamellar liposomes with the smaller pore size filters.

Example 7

In Vivo Distribution of Unilamellar Liposomes

This example illustrates the use of liposomes prepared in accordance with the present invention to deliver entrapped material in vivo. In particular, it illustrates for a rat model system the administration and subsequent in vivo distribution of ^{125}I -tyraminyl-inulin (^{125}ITI) containing LUVETs prepared in accordance with Example 1 above.

Tyraminyl-inulin was prepared as follows. Inulin (1.0g) was dissolved in 90.0 ml distilled H_2O and cooled to 4°C , 10 ml (fresh) 0.1 M periodic acid was added and the solution was incubated at 4°C for 15 minutes in the dark. Periodate consumption was assayed by the arsenite method indicating approximately two oxidations per inulin molecule (see Dyer, J. in Methods of Biochemical Analysis, P. Glick (Ed.), Vol. 3, p. 111, Interscience (1956)). The reaction was terminated by neutralization with $\text{Ba}(\text{OH})_2$ and the periodate and iodate salts were removed by centrifugation. To the supernatant 4.3 g NaH_2PO_4 and 0.55 g tyramine were added and the pH was adjusted to 7.5 with 1.0M HCL. Subsequently, NaBH_3CN (0.25 g) was added and the solution was stirred for 4 hr at room temperature. Remaining aldehyde groups were reduced by careful addition of 0.2 g NaBH_4 and the solution was stirred for another hour at 27°C . Aliquots (25 ml) were degassed under reduced pressure and applied to a 1.5 x 80 cm Sephadex G-25 column previously equilibrated with H_2O at 4°C . The flow rate was adjusted to 10 ml/hr and 4 ml fractions were collected. The fractions were assayed for tyramine by monitoring the absorbance at 279 nm and for sugar by employing the anthrone reagent technique (see Roe, J.H. (1955) J. Bio. Chem., 212:335-343). The sugar containing fraction eluted in the void volume and had a

-36-

constant tyramine:inulin mole ratio of 0.6. The adduct was completely separated from the free tyramine and other salts as determined by rechromatography on Sephadex G-25. The peak fractions were lyophilized giving an 80% yield, based on inulin.

5 The tyraminyl-inulin adduct was iodinated as follows. 2.5 mg of the tyraminyl-inulin adduct were dissolved in 0.2 ml HEPES (20 mM), NaCl (145 mM) pH 7.4 (HEPES buffered saline; HBS) and placed in a 1.5 ml stoppered vial in which 40 ug iodogen had been previously deposited from 300 uL CHCl_3 . Then carrier free Na^{125}I (4
10 mCi, 100 mCi/ml) was added and the reaction allowed to proceed for 45 min at room temperature. The solution was then transferred to a vessel containing 10 ul 0.1 M $\text{Na}_2\text{S}_2\text{O}_5$, 0.05 M KI which was then applied to a G-25 column (1 x 20 cm) equilibrated with HBS. Fractions (0.5 ml) were collected and the ^{125}I containing
15 fractions eluting in the void volume (2.5 ml) were pooled. The resulting ^{125}I -tyraminyl-inulin (^{125}ITI) solution routinely contained 1 uCi/uL ^{125}I , where less than 0.01% was in the free iodide form (less than 0.01% was CHCl_3 extractable when made to 1.2% H_2O_2 and 0.4% KI) and over 99% of the material eluted as one peak in
20 the void volume on re-chromatography employing Sephadex G-25. In all studies the material was used within 2 weeks of production.

Liposomes loaded with ^{125}ITI were prepared in accordance with the procedures described above. Specifically, 30 umol egg phosphatidylcholine (EPC) and 30 umol cholesterol were dried down
25 from CHCl_3 . The resulting lipid film was dispersed in 1 ml HBS containing 1 mCi ^{125}ITI by vortex mixing. The multilamellar systems thus produced were then extruded 10 times through two (stacked) polycarbonate Nuclepore filters (100 nm pore size) under N_2 pressure (200-400 psi). Aliquots (0.1 ml) of the LUVETs were
30 applied to an Ultrogel Ac34 column (1 ml) previously equilibrated with HBS. The lipid containing fractions were pooled and rechromatography indicated that more than 97% of the ^{125}ITI was "trapped" in the vesicles. The resulting liposome preparation had a trap volume of 0.9 ul/umol phospholipid as calculated from lipid
35 phosphate analysis and entrapped ^{125}ITI (see Fiske, C.H. and Subbarow, Y. (1925) J. Biol. Chem., 66:375-379). The average

-37-

radius of these vesicles was 70 nm. The LUVETs containing ^{125}I TI were diluted to 0.5 μmol phospholipid in 200 μl of HBS, stored at 4°C and used within 2 days of preparation.

5 The LUVETs were administered to female Wistar rats (150-200g), which were fed ad libitum prior to and during the experiments, by lightly anesthetizing the animals with ether and then injecting 200 μl HBS containing approximately 0.5 μCi ^{125}I TI encapsulated in LUVETs (0.5 μmol phospholipid) via the tail vein. The rats were allowed to recover in metabolic cages where the
10 urine and feces were collected. At various times post injection the rats were anesthetized with ether and sacrificed by exsanguination via the vena cava. Blood was collected in a syringe containing 200 μL 200 mM EDTA and recovery was approximately 85% assuming 4.9 ml blood/100 g rat. The heart, liver,
15 lung, spleen and kidney were removed and the urine remaining in the bladder was collected. The carcass was then dissolved in 200 ml 9 M NaOH at 70°C overnight. Aliquots of carcass digest and samples of tissues were then assayed for the presence of ^{125}I .

Figure 10 illustrates the clearance from the circulation of
20 the LUVETs and the subsequent appearance of inulin in the urine. As shown in that figure, the encapsulated material in the circulation is initially rapidly reduced to approximately 40% of the injected levels, and thereafter decays with a much longer half-life (approximately 3 hr). Further, only 30% of the injected
25 dose is eventually found in the urine even after 3 days. This latter result clearly indicates tissue uptake and retention of LUVET encapsulated ^{125}I TI.

The actual tissue distributions are shown in Figure 11 where approximately 50% of the in vivo ^{125}I TI is accumulated by the liver,
30 er, approximately 10% by the spleen and the rest is found in the carcass. Less than 3% ^{125}I TI was found in the heart, lung and kidney at any time post injection (data not shown).

The tissue distributions observed are similar to those previously observed with liposomes produced by other methods (see, for
35 example, Abra, R.M. and Hunt, C.A. (1981) Biochim. Biophys. Acta, 666, 493-503), thus demonstrating that the liposomes of the

-38-

present invention are equivalent with regard to in vivo behavior to prior art liposomes.

Example 8

Solvent Free Production of Liposomes

5 This example illustrates the production of liposomes directly from a lipid powder or pellet and buffer without the use of any solvents or other extraneous materials.

One hundred mg of EPC, prepared as described above, was spooned into a test tube, 1.0 ml HEPES buffer was added, and the
10 mixture was incubated at 20°C for 10 minutes. The mixture was briefly vortexed mixed for 2 minutes, followed by 5 minutes waiting time, followed by 2 minutes vortexing, and the resulting solution added to the pressure chamber of the apparatus of Figure 1A, which had been fitted with two stacked polycarbonate filters
15 having a pore size of 100 nm. The solution was extruded through the filters ten times at a temperature of 20°C. The pressures employed were on the order of 200-300 psi, and the resulting flow rates were on the order of approximately 30 ml/min.

Freeze fracture micrographs of the resulting product were
20 prepared following the procedures described above. The product was found to be a homogeneous population of substantially unilamellar liposomes having a mean diameter of approximately 70 nm as measured by freeze fracture. If desired, this mean diameter can be increased using the freeze-thaw procedures of Example 4
25 above.

The procedures described above were repeated using 200 nm filters, instead of 100 nm filters. In this case, pressures on the order of 100 psi were used, again resulting in flow rates of approximately 30 ml/min. Again, a homogeneous population of
30 liposomes was produced, but in this case a substantial portion of the population was multilamellar, rather than unilamellar. The mean diameter of this population was approximately 168 nm, as measured by quasi-elastic light scattering using a Nicomp Model 200 Laser Particle Sizer (Nicomp Instruments, Inc., Santa Barbara,
35 California).

-39-

Example 9Preparation of A Substantially Unimodal Population of Liposomes

This example illustrates the preparation of a population of liposomes having a substantially unimodal distribution.

5 Large multilamellar vesicles (MLVs) were prepared by the conventional process as follows. First, EPC prepared as described above was dissolved in chloroform and dried down and deposited as a film on the inside of a test tube. The MLVs were then formed by simply adding an aqueous buffer of 150 mM NaCl, 20 mM HEPES, pH
10 7.5, to the test tube and hydrating the lipid by vortex mixing.

The resulting MLV dispersion was then transferred into the pressure chamber of the apparatus shown in Figure 1A, equipped with two stacked standard 25 mm polycarbonate filters having a pore size of 200 nm (Nuclepore, Inc., Pleasanton, California, Catalog # 110606). The dispersion was extruded through the filters
15 25 times at a temperature of 20°C. The pressures employed were on the order of 100 psi, and the resulting flow rates were on the order of 30 ml/min. The sizing procedure was completed in less than approximately 15 minutes, and the resulting liposomes were
20 found to be substantially intact, notwithstanding their many passes through the filters.

The size distribution of the population at the end of the 25 passes was determined using the quasi-elastic light scattering technique described above. The results are shown in Table II,
25 infra.

As shown in the table, the population had a χ^2 value of 1.42 indicating that a good fit was achieved by a second order polynomial, and thus that the diffusion coefficients of the vesicles had a unimodal Gaussian distribution. The mean diameter
30 calculated for this population is 168 nm, i.e., about 15% smaller than the 200 nm pore size used for extrusion, and the standard deviation about the mean is a relatively narrow 55 nm.

-40-

Example 10Preparation of Liposomes Using A Sequence of Polycarbonate Filters

This example demonstrates the importance of using filters of a constant pore size, as opposed to a sequence of filters of decreasing pore sizes, to obtain a population of liposomes having a substantially unimodal size distribution.

MLVs were prepared as in Example 9, but instead of being extruded 25 times through filters having the same pore size, they were extruded once through a series of filters having the following pore sizes: 1000 nm, 800 nm, 600 nm, 400 nm and 200 nm (Nuclepore, Inc., Pleasanton, California, Catalog Nos. 110610, 110609, 110608, 110607, and 110606). As in Example 9, the apparatus of Figure 1A was used, equipped in this case with just a single filter for each filter size. The pressures, flow rates and processing temperature were the same as in Example 9.

The size distribution of the liposomes prepared in this manner, as determined by quasi-elastic light scattering, is shown in Table II. In this case, a huge χ^2 value of 368 was calculated, which means that a second order polynomial did not fit the data, and thus that the diffusion coefficients of the liposomes do not have a unimodal Gaussian distribution.

Comparing this result with the results for Example 9 clearly establishes that multiple passes of liposomes through filters of a constant pore size surprisingly produce a materially different size distribution from that produced by passage of the same type of liposomes through a series of filters of decreasing pore size.

Although specific embodiments of the invention have been described and illustrated, it is to be understood that modifications can be made without departing from the invention's spirit and scope. For example, the invention can be practiced with a variety of membrane forming materials and encapsulatable solutes other than those illustrated in the examples. Similarly, various types of apparatus other than that illustrated herein can be used to practice the present invention. In particular, because each of its steps is easily controllable, the method of the present invention is especially suited for implementation in a totally

-41-

automated manner, and such implementation is specifically included within the scope of the invention. Along these same lines, other types of equipment can be used to obtain autocorrelation functions, and other numerical approaches can be used to determine
5 if the autocorrelation function is of the type that would be generated from a Gaussian distribution. The scope of the invention as defined in the appended claims is intended to cover these and other variations.

10

15

20

25

30

35

-42-

TABLE I

Physical Characteristics of Vesicles Produced by Extrusion of
Various Lipid Mixtures through Filters with a Pore Size of 100 nm

LIPID	% INTENSITY*	MEAN DIAMETER ±S.D. (nm)	MEAN TRAPPED VOLUME** ±S.D. (ul/umole)
EPC	48	71±24	1.1±0.1 (64)
SPC	48	70±23	1.2±0.2 (13)
EPC/SPS (2:1)	46	73±25	1.5 (2)
SPC/SPS (2:1)	ND	73±20	2.4 (2)
SPE/SPS (2:1)	ND	79±36	2.0 (2)
SPS	ND	ND	2.3 (2)
EPS	ND	ND	2.2 (2)
EPC (Freeze-thaw)	51	77±16	2.2±0.5 (17)
SPC (Freeze-thaw)	48	94±26	2.2±0.1 (12)
EPC (Octylglucoside)	49	ND	1.2±0.1 (3)
EPC (REV)	50	ND	1.2 (2)

* Intensity of ^{31}P -NMR signal remaining in the presence of 5 mM Mn^{2+} .

** ul/umole phospholipid (number of experiments in parenthesis)

-43-

TABLE II

Properties of Liposome Populations
Measured By Quasi-Elastic Light Scattering*

	Constant Pore Size	Decreasing Pore Size
Mean Diameter (nm)	168	630
χ^2	1.42	368
Standard Deviation (nm)	55	**
Lipid Concentration (mg/ml)	10	10

* The values for mean diameter, χ^2 and standard deviation were determined using a Nicomp Model 200 Laser Particle Sizer (Nicomp Instruments, Inc., Santa Barbara, CA 93111). The following input parameters were used: temperature -- 20°C; viscosity -- 1.002 centipoise; index of refraction -- 1.330; laser wavelength -- 632.8 nm; and sine of angle/2 -- 0.7070. For both sets of measurements, the instrument was used in the delayed baseline mode. For the constant pore size measurements (Example 9), a run time of 6.33×10^6 msec was used, which produced a total count value of 9.08×10^8 , and a channel width of 20 usec was used, which gave an autocorrelation function having 1.98 decays. For the decreasing pore size measurements (Example 10), a run time of 6.58×10^6 msec was used, which gave a total count value of 8.01×10^8 , and a channel width of 70 usec was used, which gave an autocorrelation function having 1.85 decays.

** Not reported in view of χ^2 value (see text).

-44-

What is claimed is:

1. A method for reducing the lamellarity of a population of liposomes comprising repeatedly passing the liposomes under pressure through a filter which has a pore size equal to or less than about 100 nm.
2. The method of Claim 1 wherein the population of liposomes is passed through the filter more than two times.
3. The method of Claim 1 wherein the filter has straight through channels.
4. The method of Claim 3 wherein the filter is a polycarbonate filter.
5. A population of liposomes whose lamellarity has been reduced by the method of Claim 1.
6. A method for preparing a population of substantially unilamellar liposomes comprising the steps of:
 - a) preparing multilamellar liposomes; and
 - b) repeatedly passing the liposomes under pressure through a filter which has a pore size less than or equal to about 100 nm.
7. The method of Claim 6 wherein the liposomes are passed through the filter more than two times.
8. The method of Claim 6 wherein the filter has straight through channels.
9. The method of Claim 8 wherein the filter is a polycarbonate filter.
10. The method of Claim 6 including the additional step of subjecting the liposomes to a freeze-thaw cycle to increase their trapped volumes.
11. A population of substantially unilamellar liposomes prepared by the method of Claim 6.
12. A method for preparing liposomes without the use of solvents, detergents or other extraneous materials comprising the steps of:
 - a) preparing a mixture of a lipid powder or pellet and an aqueous buffer; and
 - b) repeatedly passing the mixture under pressure through a filter.

-45-

13. The method of Claim 12 wherein the filter has straight through channels.

14. The method of Claim 13 wherein the filter is a polycarbonate filter.

5 15. The method of Claim 12 including the additional step of subjecting the liposomes to a freeze-thaw cycle to increase their trapped volumes.

16. The method of Claim 12 wherein the filter has a pore size less than or equal to about 100 nm, the mixture is passed
10 through the filter more than two times, and the resulting liposomes are substantially unilamellar.

17. Liposomes prepared by the method of Claim 12.

18. A population of liposomes having a substantially unimodal distribution about a mean diameter greater than 50 nm.

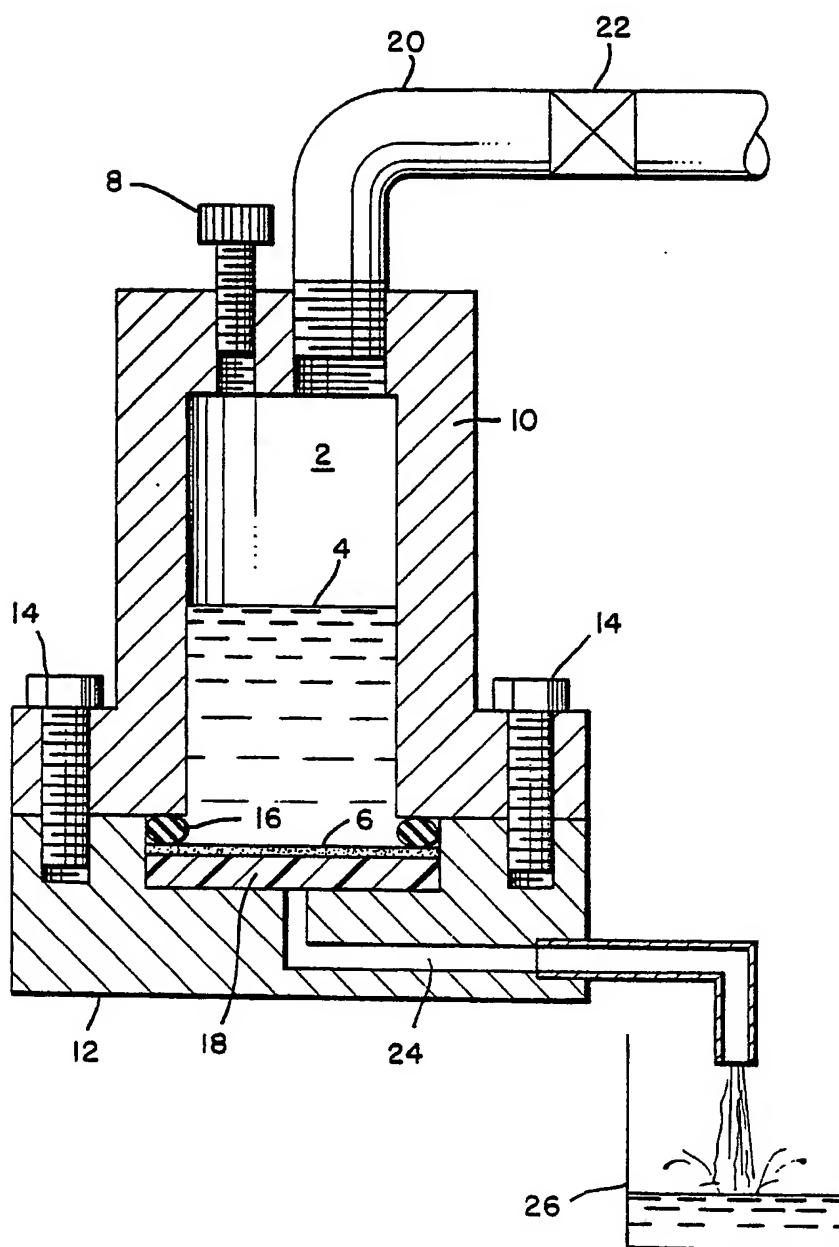
15 19. The population of Claim 18 wherein the natural logarithm of the autocorrelation function of the time dependent fluctuations in the intensity of light scattered by a sample of the population during illumination of the sample with coherent light can be closely fit by a second order polynomial.

20 20. A method for increasing the homogeneity of a population of liposomes comprising repeatedly passing the liposomes through one or a plurality of filters, all of which have the same pore size, the number of passes through the filter or filters being sufficient to produce a substantially unimodal distribution of the
25 diameters of the liposomes about a mean value.

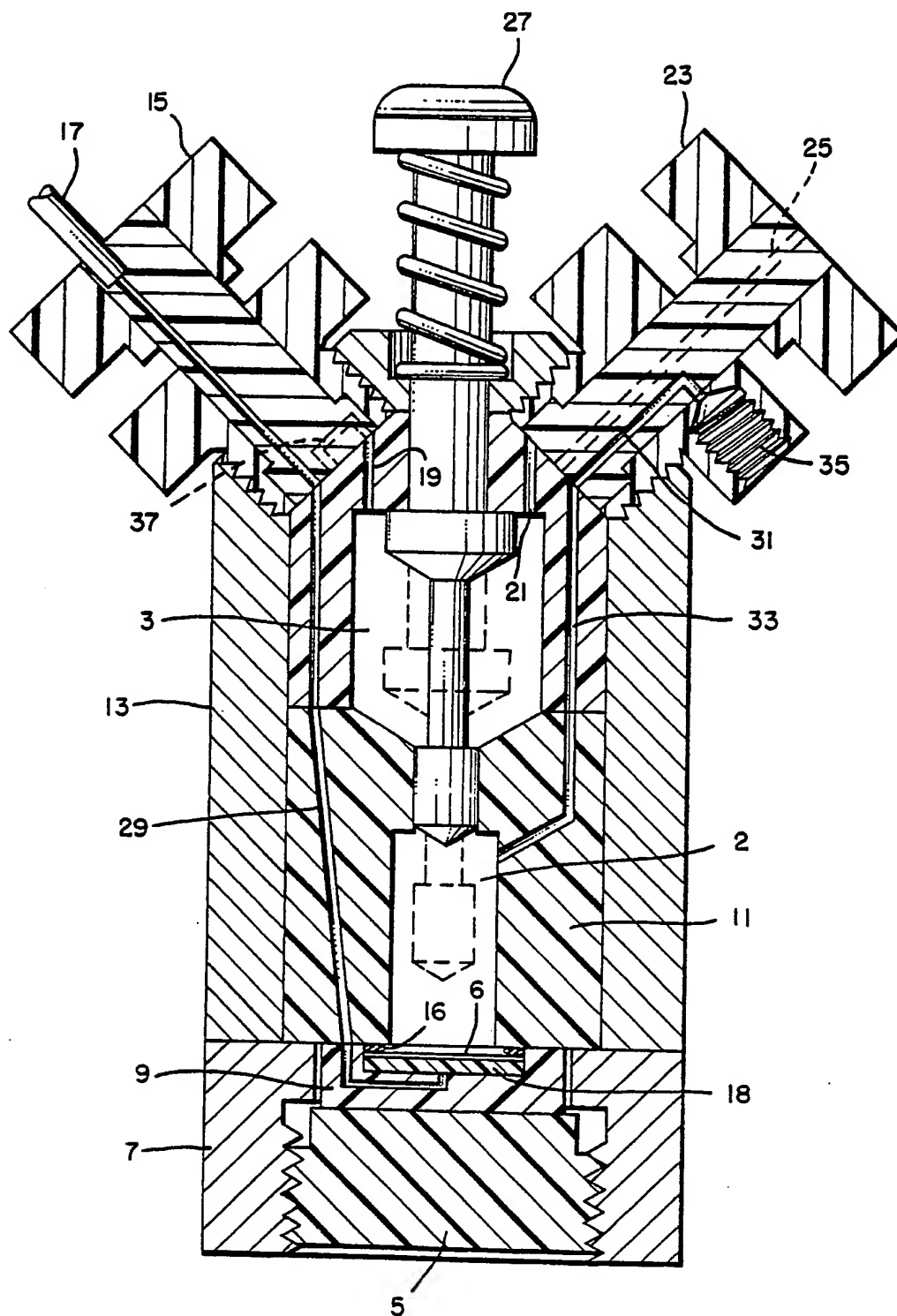
30

35

1/9

FIG. 1A.

2/9
FIG. 1B.



3/9

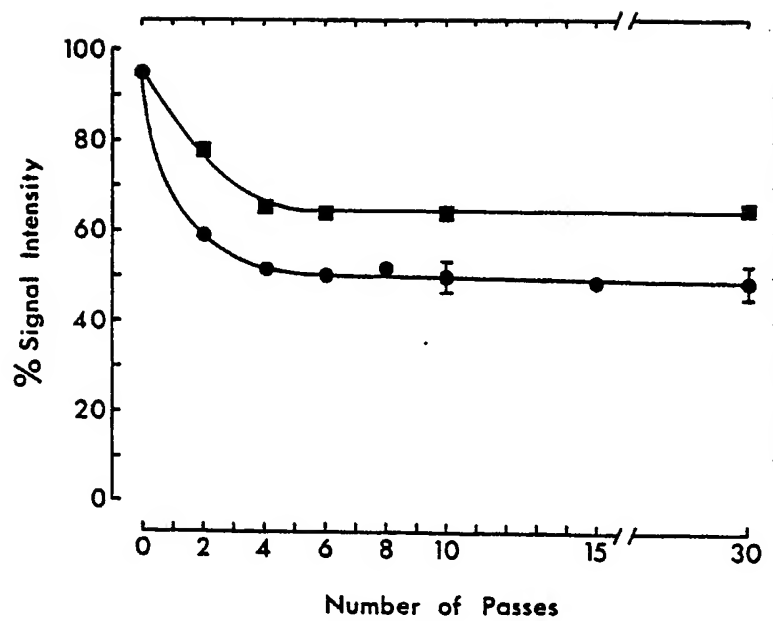
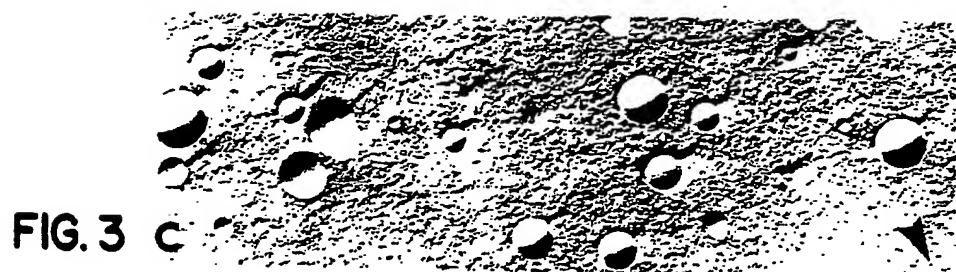
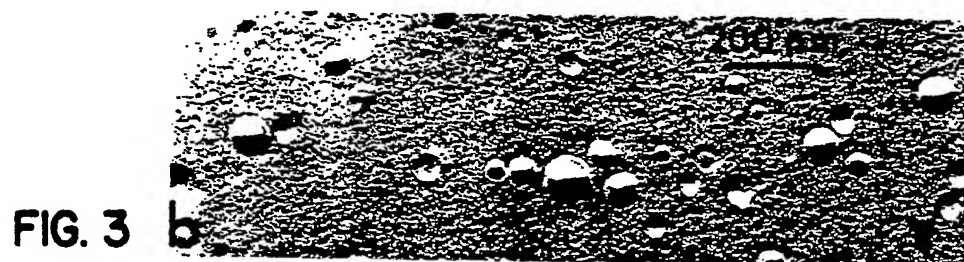
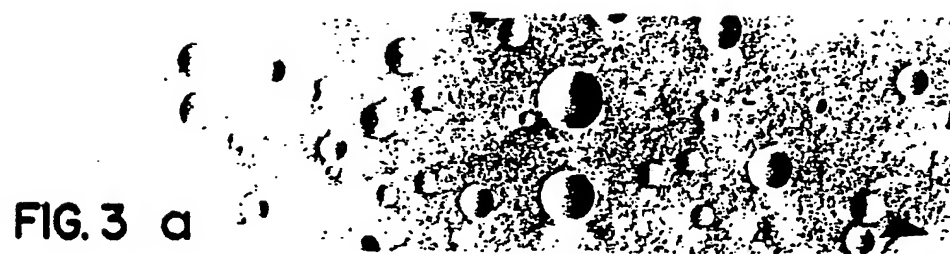
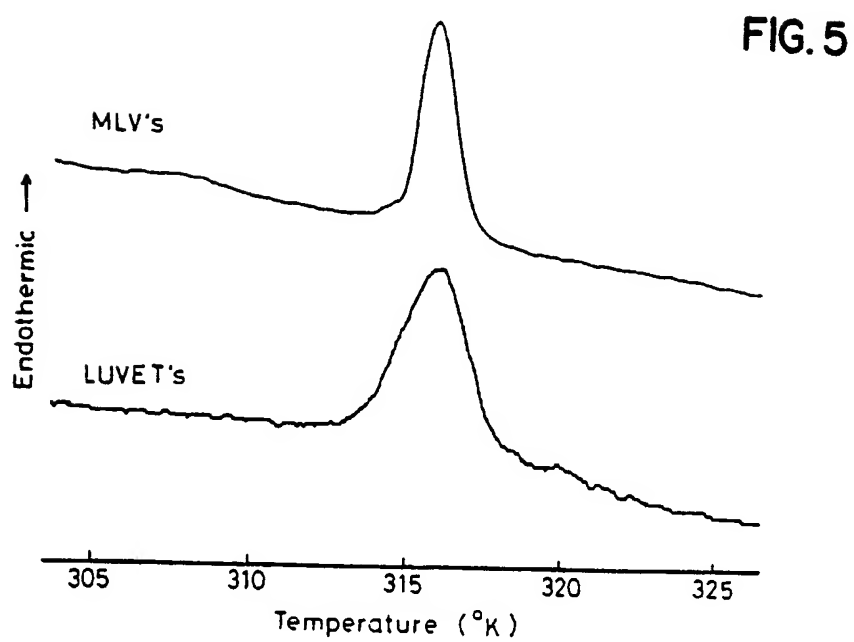
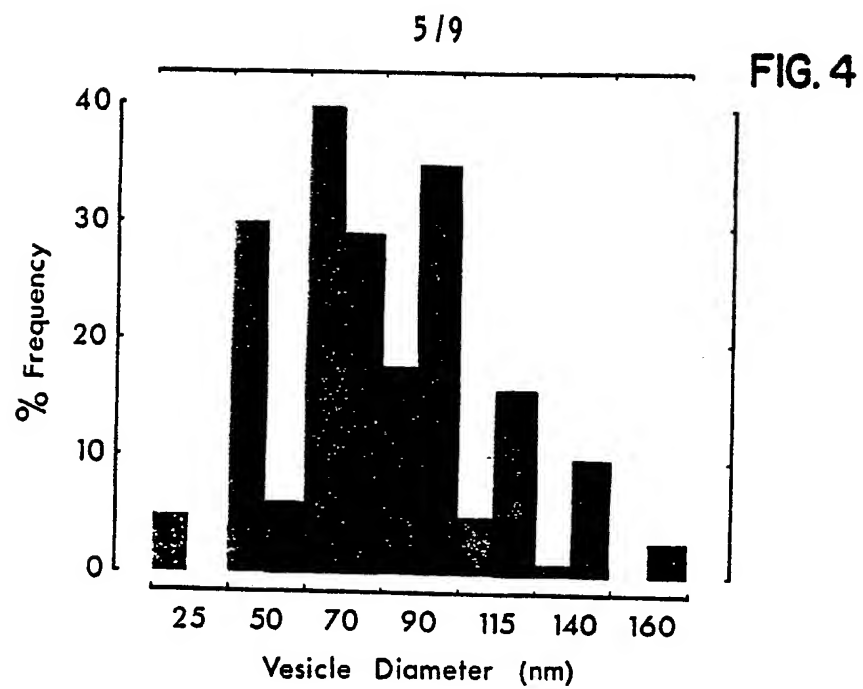


FIG. 2

4/9





6/9

FIG. 6

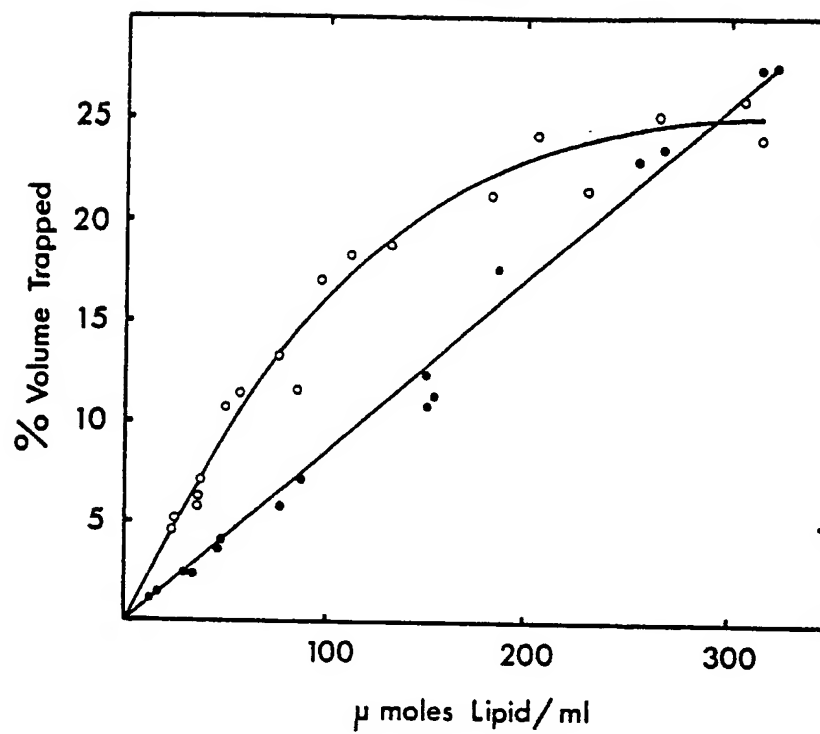
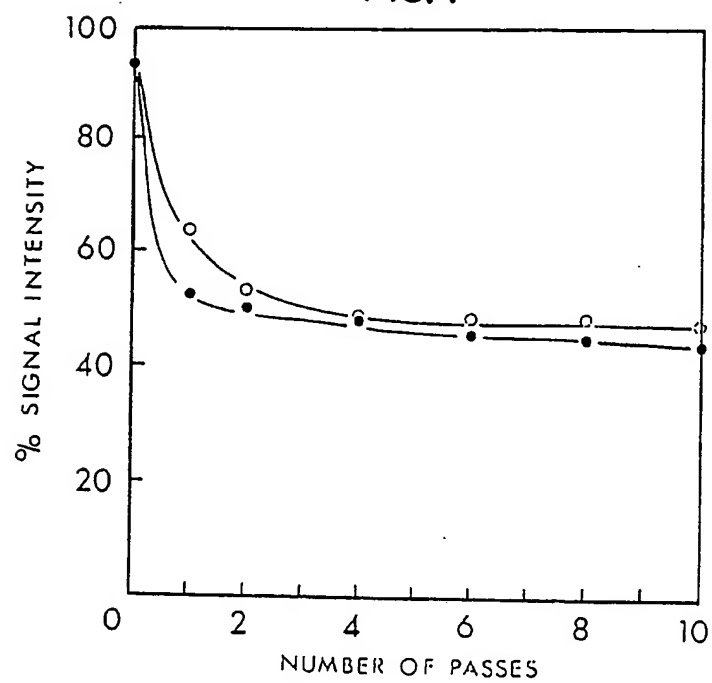


FIG. 7



719

FIG. 8 a

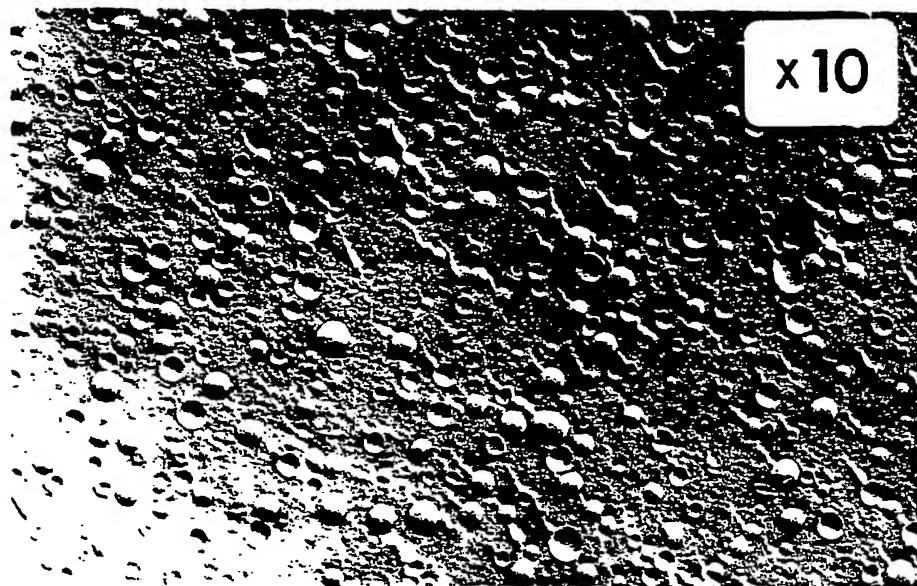
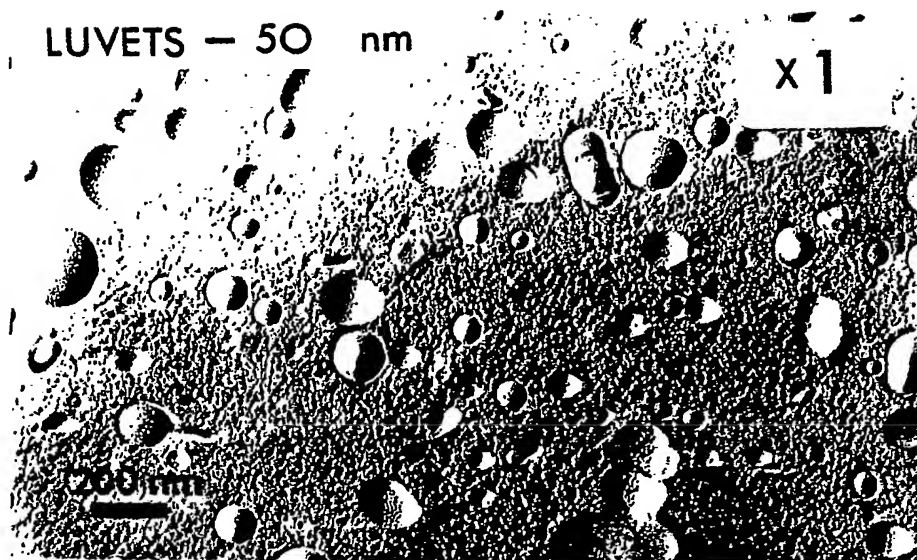


FIG. 8 b

8/9

FIG. 9 a

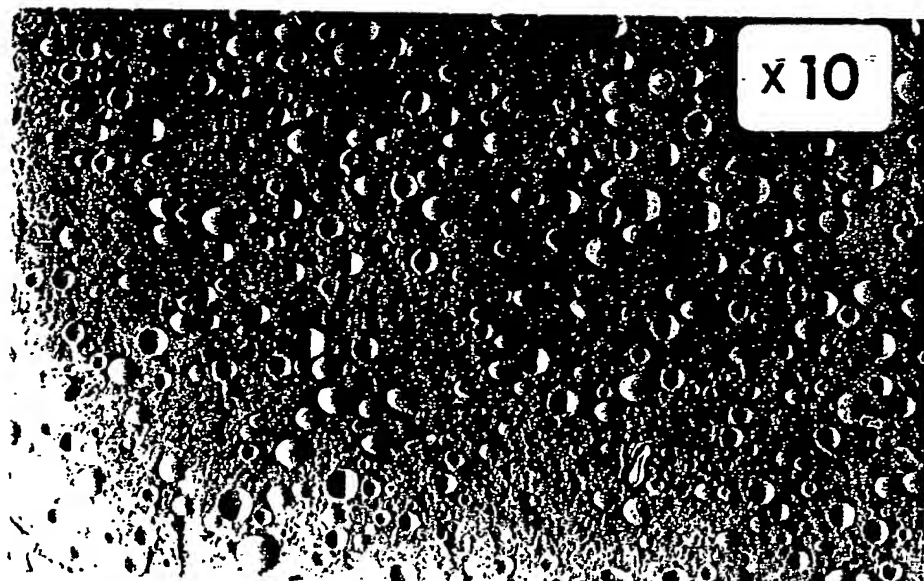
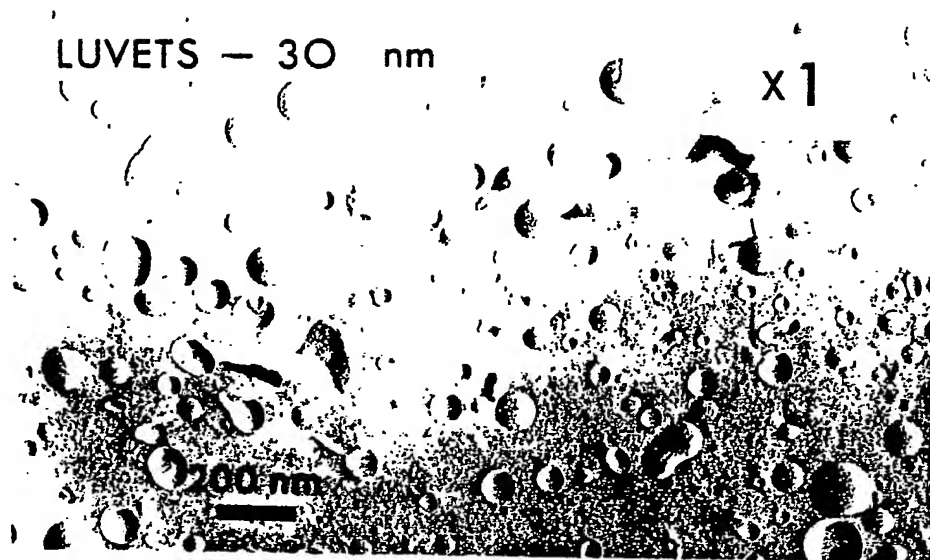


FIG. 9 b

9/9

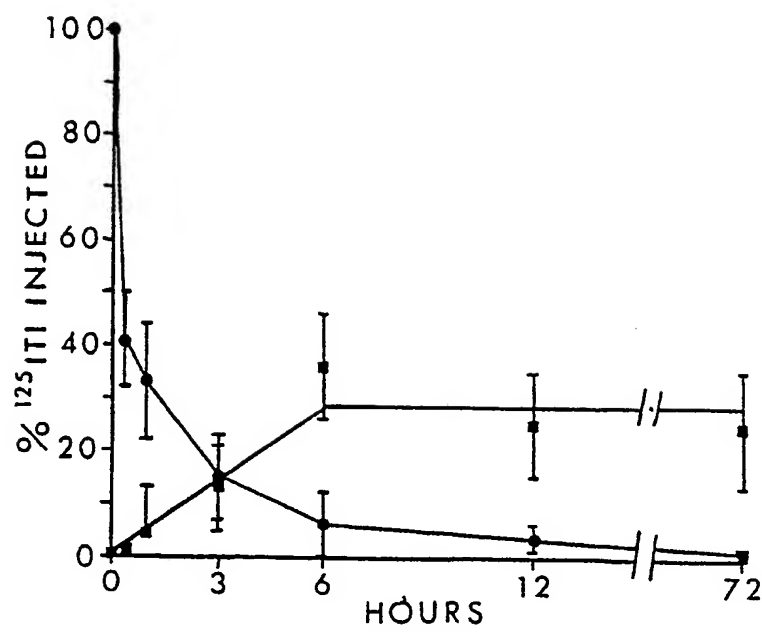


FIG. 10

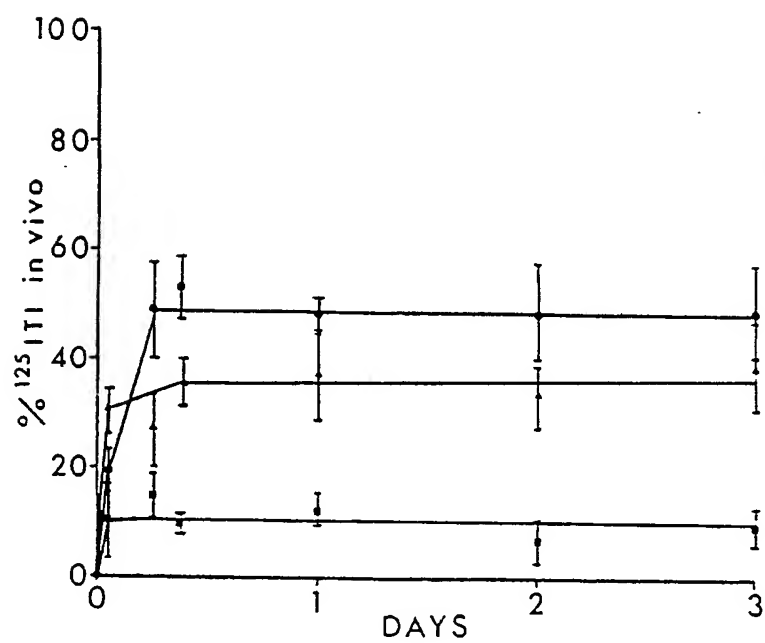
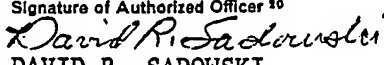


FIG. 11

INTERNATIONAL SEARCH REPORT

International Application No PCT/US85/01161

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) ³		
According to International Patent Classification (IPC) or to both National Classification and IPC		
INT. CL. <u>B01D 13/00</u>		
U.S. CL. 210/651, 805, 806; 436/829		
II. FIELDS SEARCHED		
Minimum Documentation Searched ⁴		
Classification System	Classification Symbols	
U.S.	210/637, 650, 651, 805, 806 436/829	
Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched ⁵		
III. DOCUMENTS CONSIDERED TO BE RELEVANT ¹⁴		
Category ⁶	Citation of Document, ¹⁵ with indication, where appropriate, of the relevant passages ¹⁷	Relevant to Claim No. ¹⁸
Y	Journal of Pharmaceutical Science, Vol. 71 No. 7 Issued July, 1982, M. E. Bosworth et al, Liposome Dialysis for Improved Size Distributions, pages 806- 812, see page 808.	1-20
Y	International Journal of Pharmaceutics, 17, Issued 1983, M. Morii et al, Size and permeability of Liposomes extruded through polycarbonate membranes, pages 215 to 224, see page 217.	1-20
Y	Biochimica et al Biophysica Acta, 557, Issued 1979, Olson et al, Preparation of Liposomes of Defined Size Distribution by Extrusion Through Polycarbonate Membranes, pages 9 to 23, see page 11.	1-20
<div style="display: flex; justify-content: space-between;"> <div style="width: 45%;"> <p>[*] Special categories of cited documents: ¹⁶</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> </div> <div style="width: 45%;"> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"&" document member of the same patent family</p> </div> </div>		
IV. CERTIFICATION		
Date of the Actual Completion of the International Search ³	Date of Mailing of this International Search Report ³	
11 JULY 1985	25 JUL 1985	
International Searching Authority ¹	Signature of Authorized Officer ²⁰	
ISA/US	 DAVID R. SADOWSKI	

THIS PAGE BLANK (USPTO)

**This Page is Inserted by IFW Indexing and Scanning
Operations and is not part of the Official Record**

BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images include but are not limited to the items checked:

- ☒ **BLACK BORDERS**
- ☐ **IMAGE CUT OFF AT TOP, BOTTOM OR SIDES**
- ☐ **FADED TEXT OR DRAWING**
- ☐ **BLURRED OR ILLEGIBLE TEXT OR DRAWING**
- ☐ **SKEWED/SLANTED IMAGES**
- ☒ **COLOR OR BLACK AND WHITE PHOTOGRAPHS**
- ☐ **GRAY SCALE DOCUMENTS**
- ☐ **LINES OR MARKS ON ORIGINAL DOCUMENT**
- ☐ **REFERENCE(S) OR EXHIBIT(S) SUBMITTED ARE POOR QUALITY**
- ☐ **OTHER:** _____

IMAGES ARE BEST AVAILABLE COPY.

As rescanning these documents will not correct the image problems checked, please do not report these problems to the IFW Image Problem Mailbox.

THIS PAGE BLANK (USPTO)